2005

Glucose transporter 2 is likely to play a role in the brain glucose sensing

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GLUCOSE TRANSPORTER 2 IS LIKELY TO PLAY A ROLE IN THE BRAIN GLUCOSE SENSING

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Human Ecology

by
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May 2005

DEDICATION

I would like to dedicate this to my family and my major professor Dr. Martin.

ACKNOWLEDGEMENTS

I will forever be thankful to Dr Martin, who has been so patiently and yet strictly mentoring me through my doctoral study.

I would like to thank all those (colleagues in Dr Martin's laboratory and others in Pennington Biomedical Research Center) who have helped me during my working on this dissertation. Particularly, I would like to thank Dr David S. Roane and Dr. Kichoon Lee for their mentoring and Xiaochun Xi for her technical assistance.

I would like to thank Dr Roy J Martin, Dr Kichoon Lee, Dr David S Roane, Dr Maren Hegsted, Dr Yan Chen, Dr J Marcos Fernandez and Dr Joseph Taboada, for their valuable comments on this manuscript.

And lastly, I would like to thank my parents and my brother; I would not be able to overcome all the difficulties during this graduate study without their continuous supports and encouragements.

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ABSTRACT

It has been proposed that the glucose sensing mechanism in the hypothalamus and hindbrain is similar to pancreatic β cells, and brain glucose sensing may be involved in the regulation of food intake. For the first part of the dissertation, it is proposed that molecules involved in β cell glucose sensing, including glucokinase (GK), glucose transporter GLUT2, sulfonylurea receptor-1 (SUR1), glucagon-like peptide-1 receptor (GLP-1R), and the feeding-related neuropeptide Y (NPY), are colocalized in specific areas in the hypothalamus and hindbrain. GK, GLUT2, SUR1, GLP-1R and NPY mRNA expression in ten discrete brain areas were quantified by real time RT-PCR, which will serve as an initial step for the next functional study.

The second part of the dissertation has been focused on GLUT2 only and it is proposed that brain GLUT2 may play a role in the central glucose sensing, specifically, brain GLUT2 is regulated by energy / glucose status, and overexpression of GLUT2 in neuronal cells will alter cellular energy status and feeding-related neuropeptide expression. Under three conditions: *in vivo*, *ex vivo* and *in vitro*, GLUT2 mRNA was significantly upregulated in the area postrema (AP) in the two-week 50% underfed rats, and by 1 mM glucose in rat area postrema / nucleus of the solitary tract (AP/NTS) tissue culture as well as in N1E-115 neuroblastoma cell culture. Next, rat liver GLUT2 were overexpressed in GT1-7 neuroblastoma cells. Compared with control cells, GLUT2 overexpression resulted in significantly increased cellular ATP levels at 5 mM or higher glucose concentrations, greater inhibition of AgRP mRNA by 25 mM glucose, and attenuated AgRP mRNA stimulation by 2DG. In summary, brain GLUT2 mRNA is upregulated by low energy and low glucose status; overexpression of GLUT2 in neuronal cells results in higher cellular energy status and

greater suppression of hunger signals at high glucose levels or during glucoprivation. The conclusion is that brain GLUT2 is likely to play a role in the central glucose sensing and may be involved in the regulation of food intake.

CHAPTER 1

INTRODUCTION

Obesity is a condition characterized by excessive body fat. The prevalence of obesity has increased dramatically over the past few decades. The 1988-1994 NHANES study showed that an estimated 56% of U.S. adults were either overweight (33%) or obese (23%) (NHANES 1996). The initial results from the 1999 NHANES indicated that approximately 61% of U.S. adults were overweight (35%) or obese (26%) (NHANES 1999). Obesity is not only a chronic disease by itself, but also an independent risk factor for many other chronic diseases such as cardiovascular diseases, type II diabetes, hypertension, hyperlipidemia and cancer (Bethesda 1998). Obesity and its associated diseases are costly; some of which are life threatening. Therefore, it is important to investigate the mechanism of obesity development in order to find effective ways to treat and prevent obesity and to help people maintain normal body weight.

The current understanding of the etiology of obesity is incomplete. Many factors, including both genetics and environment, contribute to the development of obesity (Bethesda 1998). When the interactions among these factors result in the long-term energy intake being greater than the energy expenditure, overweight and obesity will develop (Wolf and Tanner 2002).

Energy intake is associated with appetite and food intake, i.e. feeding behavior; energy expenditure is associated with physical activity and heat production. The brain plays an essential role in sensing energy status and in maintaining energy homeostasis (Schwartz, Woods et al. 2000). Glucose is the primary fuel for the brain tissue; the glucose sensing mechanism in the brain may play an important role in the maintenance of energy homeostasis

and glucose homeostasis (Levin, Dunn-Meynell et al. 1999; Penicaud, Leloup et al. 2002). Defect or dysfunction in the brain glucose sensing mechanism may result in abnormal energy status, leading to weight gain or weight loss. A long-term weight gain will lead to obesity.

This dissertation is mainly focused on the mechanisms underlying brain glucose sensing and how signals from glucose fluctuation were translated to regulate feeding-related neuropeptide expression. Previous studies suggest that the hypothalamus and the area postrema and nucleus of the solitary tract (AP/NTS) in the hindbrain are critical in brain glucose sensing and in the control of feeding behavior (Ritter, Slusser et al. 1981; Penicaud, Pajot et al. 1990; Navarro, Rodriquez de Fonseca et al. 1996; Lynch, Tompkins et al. 2000; Schuit, Huypens et al. 2001). Glucose transporter 2 (GLUT2), one of the glucose sensors in the pancreatic β cells, is also expressed in the hypothalamus and hindbrain AP/NTS. GLUT2 in the brain may be regulated by energy status (Zhou, Roane et al. 2003; Bogacka, Roane et al. 2004) and blocking of brain GLUT2 may affect energy homeostasis (Leloup, Orosco et al. 1998; Wan, Hulsey et al. 1998). Until now, the regulation of brain GLUT2 and the role of GLUT2 in the brain are not completely understood.

The hypothesis for this work is that brain GLUT2 is likely to play a role in the central glucose sensing. To test the hypothesis, firstly, GLUT2 was localized in discrete nuclei in the hypothalamus and the hindbrain. Secondly, the regulation of brain GLUT2 was investigated by three experiments to examine GLUT2 mRNA responses to low energy status in underfed rat brain, and to different glucose status in *ex vivo* cultured brain tissue and in N1E-115 neuroblastoma cell line. Lastly, GLUT2 was overexpressed in GT1-7 neuroblastoma cell line, and ATP and agouti-related peptide (AgRP) mRNA responses to glucose and 2-deoxyglucose (2DG) were examined in these cells. There is no known report of the effect of low energy status on GLUT2 levels in the brain. There is no known report showing direct

evidence of GLUT2 response to glucose status in *in vitro* and *ex vivo*. There is no known report of the neuropeptide response to GLUT2 overexpression in neuronal cell line. This dissertation will contribute to the understanding of the regulation of brain GLUT2 by energy and glucose status, and the role of brain GLUT2 in the central glucose sensing in the aspect of controlling ATP level and hunger signals like AgRP in neuronal cells.

CHAPTER 2

REVIEW OF LITERATURE

Central Regulation of Food Intake

Hypothalamic Control of Energy Homeostasis

As reviewed by Schwartz et al (Schwartz, Woods et al. 2000), the hypothalamus plays a critical role in the control of energy homeostasis. A number of feeding related neuropeptides are expressed in the specific nuclei in the hypothalamus. Based on their effect on energy homeostasis, these neuropeptides can be divided into two groups: orexigenic and anorexigenic. The orexigenic neuropeptides stimulate energy intake and inhibit energy expenditure; and the anorexigenic neuropeptides exert opposite effects. Examples of orexigenic peptides are neuropeptide Y (NPY), agouti-related peptide (AgRP), melanin-concentrating hormone (MCH), orexins A and B (ORX). Examples of anorexigenic peptides are α-melanocyte-stimulating hormone (α-MSH), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH). NPY and AGRP are co-localized in the arcuate NPY/AGRP neurons, α-MSH and CART are co-localized in the arcuate POMC/CART neurons, MCH and ORX neurons are localized in the lateral hypothalamus (LH), CRH and TRH neurons are localized in the paraventricular nucleus (PVN). Neuropeptides or receptor knockout studies indicate that some neuropeptides are redundant and can be compensated whereas others are more essential. For example, NPY knockout mice, AgRP knockout mice, or NPY/AgRP double knockout mice exhibits normal food intake and body weight (Qian, Chen et al. 2002). On the other hand, mice lacking MCH are hypophagic and lean (Shimada, Tritos et al. 1998), and mice lacking α-MSH receptor MC4R are hyperphagic and obese (Huszar, Lynch et al. 1997).

In the hypothalamus, the first order neurons in the arcuate integrate multiple inputs from peripheral (such as leptin and insulin) and the hindbrain, then send out signals to second order neurons in the PVN and LH, which will stimulate or inhibit feeding. Second order neurons also accept inputs from peripheral and hindbrain, and there are feedback interactions between the first and second order neurons (Schwartz, Woods et al. 2000). Taken together, the hypothalamic control of energy homeostasis involves inter-dependent interactions between the hypothalamus and peripheral and hindbrain, and multiple neuropeptides serve as the effecter as well as regulator in this system to regulate energy homeostasis.

AMPK in the Hypothalamic Regulation of Feeding

AMP-activated protein kinase (AMPK) has been known as a cellular energy sensor, and an increase in AMP to ATP ratio induces AMPK phosphorylation and activates AMPK (Hardie, Carling et al. 1998). AMPK has been proposed to be an energy sensor and regulator of food intake in the hypothalamus (Andersson, Filipsson et al. 2004; Kim, Miller et al. 2004; Kim, Park et al. 2004; Landree, Hanlon et al. 2004; Lee, Li et al. 2005; Minokoshi, Alquier et al. 2004). In the hypothalamus, AMPK activity is suppressed by glucose (i.p or ICV), refeeding, leptin, and insulin; on the other hand, AMPK activity is stimulated by low glucose, 2DG and ghrelin. Activation of hypothalamic AMPK by overexpression of constitutively active AMPK or ICV injection of AMPK activator AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) increases food intake and body weight gain (Andersson, Filipsson et al. 2004; Minokoshi, Alquier et al. 2004); whereas inhibition of hypothalamic AMPK by overexpression of dominant negative AMPK or ICV injection of AMPK inhibitor compound C decreases food intake and body weight gain, or attenuates 2DG-induced feeding (Kim, Park et al. 2004; Minokoshi, Alquier et al. 2004). C75 is a synthetic fatty acid synthase inhibitor; C75 administration (both i.p. and ICV) suppresses food intake in mice (Loftus,

Jaworsky et al. 2000). In the hypothalamus, C75 inhibits AMPK phosphorylation, and the inhibitory effect of C75 on food intake can be reversed by subsequent administration of the AMPK activator AICAR; suggesting that hypothalamic AMPK mediates the inhibitory effect of C75 on food intake (Kim, Miller et al. 2004). Taken together, AMPK in the hypothalamus plays an important role in energy sensing and regulation of food intake.

AgRP-expressing Neuroblatoma Cells

Agouti related peptide (AgRP), or agouti related transcript (ART), is an orexigenic neuropeptide that robustly stimulates food intake (Schwartz, Woods et al. 2000). In mice and rats, AgRP is mainly expressed in the brain and adrenal gland, and to a less extent in the lung and testis. In the brain, AgRP expression is localized in the arcuate nucleus and median eminence (Ollmann, Wilson et al. 1997; Shutter, Graham et al. 1997). In the arcuate nucleus, AgRP is colocalized with neuropeptide Y (NPY), which is another robust orexigenic neuropeptide (Shutter, Graham et al. 1997; Hahn, Breininger et al. 1998). In the hypothalamus, AgRP mRNA expression is increased about eight to ten fold in obese mouse model (ob/ob mice, db/db mice) compared with controls (Ollmann, Wilson et al. 1997; Shutter, Graham et al. 1997). Hypothalamic AgRP mRNA is stimulated by fasting and 2DG, and inhibited by leptin (Ollmann, Wilson et al. 1997; Mizuno, Makimura et al. 1999; Mizuno and Mobbs 1999; Sergeyev, Broberger et al. 2000; Bertile, Oudart et al. 2003). Overexpression of AgRP in transgenic mice leads to obesity (Ollmann, Wilson et al. 1997). ICV injection of AgRP increases food intake, and the stimulatory effect on food intake by a single dose of AgRP injection lasts a week, implicating that AgRP is a robust stimulator of food intake (Hagan, Rushing et al. 2000). AgRP effect on food intake involves antagonism to melanocortin receptor MC3R and MC4R (Ollmann, Wilson et al. 1997); other mechanisms

including opioid receptor and calcium flux may also be involved but are not well understood to date (Hagan, Rushing et al. 2000; Hagan, Rushing et al. 2001).

Neuroblastoma cell lines have been used to investigate how energy and glucose status regulate the expression of orexigenic neuropeptides. N1E-115 cell is a mouse neuroblastoma cell line that express multiple neuropeptides and receptors, among which AgRP, MC3R, MC4R leptin OB-receptor, insulin receptor are expressed at relatively high levels (Roth, Yee et al. 2002). AgRP mRNA decreases in a dose-dependent manner in the N1E-115 cells when cultured in DMEM containing increasing concentrations of glucose (1, 2.5, 5, and 10 mM) (Lee, Li et al. 2005). In addition to the neuropeptides and receptors, GLUT2 mRNA has been detected at measurable levels by real time RT-PCR. N1E-115 cell will be a good in vitro system to examine the effect of glucose on GLUT2 mRNA expression for the purpose of this dissertation.

GT1-7 cell is an immortalized hypothalamic GnRH (gonadotropin-releasing hormone) cell line (Mellon, Windle et al. 1990). GT1-7 cells express MC4R and MC3R (Khong, Kurtz et al. 2001). GT1-7 cells also express AgRP mRNA; AgRP mRNA in GT1-7 cells decreases dose-dependently in response to an increasing glucose concentration in culture medium (Lee, Li et al. 2005). GLUT2 mRNA is not detected in GT1-7 cells by real time RT-PCR (Li, unpublished data). This cell line will be used for in vitro overexpression of GLUT2 and AgRP response to glucose and 2DG after GLUT2 overexpression will be determined.

2DG, 5TG and Glucose as Manipulators of Energy Status

2-Deoxyglucose (2DG), 5-thioglucose (5TG) and glucose have been used to manipulate glucose status in the brain. 2DG and 5TG are non-metabolizable glucose analogues. 2DG or 5TG competitively inhibits the intracellular metabolism of glucose and

induces glucoprivation. Injection of 2-DG (or 5TG) into the lateral cerebral ventricle stimulates feeding and hyperglycemia in rats; at the same dose, 2DG does not stimulate feeding when injected intravenously and subcutaneously into rats. Therefore, the increased food intake is due to the direct effect of 2DG on the brain (Miselis and Epstein 1975). 2DG stimulates feeding by induction of NPY / AgRP expression in the arcuate nucleus and MCH expression in the lateral hypothalamus (Sergeyev, Broberger et al. 2000). Central glucose injection has been used to suppress food intake in rats. Infusion of glucose in the third ventricle of the rat brain over a period of seven days reduces both food intake and body weight. This amount of glucose does not change blood glucose level, suggesting that the depressed food intake is due to the effect of glucose on the brain (Davis, Wirtshafter et al. 1981). Central administration of 2DG, 5TG or glucose can be used to manipulate brain glucose status and to change feeding behavior.

Involvement of Hypothalamus and Hindbrain in Glucoprivic Feeding

The hypothalamus is not necessary for 2DG-induced glucoprivation. 2DG directly injected into the LH and VMH fails to induce feeding in the rats (Miselis and Epstein 1975). Injection of 5TG into 61 specific hypothalamic areas fails to induce hyperglycemia and feeding in all of the areas studied, including arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, paraventricular nucleus, and lateral hypothalamic area (Ritter, Dinh et al. 2000). Taken together, hypothalamus is not the primary site for glucoprivic feeding.

The hindbrain is necessary for glucoprivic feeding. Injection of 5TG into the lateral or fourth ventricle stimulates feeding. When the aqueduct connecting the third and fourth ventricle is blocked, subsequent injection of 5TG into the lateral ventricle fails to stimulate feeding, yet injection of 5TG into the fourth ventricle still stimulates feeding (Ritter, Slusser et al. 1981; Penicaud, Pajot et al. 1990). Lesions of area postrema in the hindbrain blocked

2DG or 5TG induces glucoprivic feeding, suggesting that area postrema is a critical area for glucoprivic feeding (Contreras, Fox et al. 1982; Bird, Cardone et al. 1983).

To further identify specific areas in the hindbrain responsible for glucoprivic feeding, Ritter et al (Ritter, Dinh et al. 2000) tested glycemic and feeding response to microinjection of 5TG (24ug in 200nl) in 142 hindbrain areas. 46% of tested hindbrain areas show positive glycemic and feeding response to 5TG, with an average increase of 75.9 mg/dl in blood glucose and 2.8 g in food intake for all positive sites. The positive sites are mainly located in the ventrolateral and dorsomedial medulla (including NTS), and more in the caudal than the rostral part of hindbrain, with almost complete overlapping of the sites showing positive glycemic and feeding response. Taken together, hindbrain is necessary for glucoprivic feeding.

Brain Glucose Sensing

Brain Glucose Levels

Brain glucose level changes parallel with blood glucose level. Extracellular glucose concentration in the brain (cortex) can be measured by using a microelectrode. In normal Wistar rats, when blood glucose concentration is 7.6 mM, the corresponding glucose concentration in the brain is 2.4 mM. During hyperglycemia induced by intraperitoneal injection of glucose, the brain glucose concentration increases to 4.5 mM when blood glucose reaches 15.2 mM. During hypoglycemia induced by insulin injection, brain glucose concentration decreases to 0.16 mM when blood glucose lowers to 2.8 mM. At normoglycemia to hyperglycemia, brain glucose is about 31% of blood glucose level; at hypoglycemia, brain glucose drops to about 10% of blood glucose level (Silver and Erecinska 1994). Recordings of extracellular glucose concentration in ventromedial hypothalamus (VMH) by microdialysis show consistent results. VMH glucose is 18% of

blood glucose (1.42 mM vs. 7.7 mM) in fed rats; VMH glucose is 13% of blood glucose (0.73 mM vs. 5.8 mM) in fasted rats (de Vries, Arseneau et al. 2003). Taken together, brain glucose level is 10 % to 30% of blood glucose level, and brain glucose changes parallel with blood glucose under different glycemic conditions.

Glucose Sensing Neurons in the Hypothalamus and Hindbrain

Hypothalamus and hindbrain area postrema and nucleus of the solitary tract (AP/NTS) contain glucose-sensing neurons (Anand, Chhina et al. 1964; Oomura, Kimura et al. 1964; Mizuno and Oomura 1984; Riediger, Schmid et al. 2002). Neurons that increase firing as glucose levels increase were glucoreceptor (GR) neurons; neurons that decrease firing as glucose levels increase were glucose-sensitive neurons (GS). In the hypothalamus, GR neurons were mainly located in the ventromedial hypothalamus, and GS neurons were mainly located in the lateral hypothalamus (Oomura 1983). It is hypothesized that during hyperglycemia, increased glucose enters the neurons, being metabolized, leading to increased intracellular ATP concentration. Increased ATP inhibits ATP-sensitive K channel and activates GR neurons; increased ATP can also stimulate Na/K ATPase and inhibit GS neurons (Silver and Erecinska 1998; Song, Levin et al. 2001).

Silver et al further categorized the glucose sensing neurons in the hypothalamus (Silver and Erecinska 1998). In the lateral hypothalamic area (LHA), 33% of neurons responded change in glucose levels, of which 93 – 95 % neurons are inhibited by a rise in glucose levels, i.e. GS neurons, and only 5 – 7 % are stimulated by a rise in glucose levels, i.e. GR neurons. GS neurons are divided into three types: type I neurons (about 60%) completely stop firing when blood glucose are between 10 – 12 mM; type II neurons stop firing when blood glucose is about 17 mM; and type III neurons keep firing at a lower rate when blood glucose is higher than 17 mM. All VMH neurons in this study increase firing

when blood glucose increases, i.e. GR neurons, most of which keep high firing rates when blood glucose was higher than 15 mM. When blood glucose is lowered to 3 – 4 mM, more than 90% of VMH GR neurons stop firing.

Song et al studied VMH glucose sensing neurons from brain slices and defined 5 types of neurons in the VMH (Song, Levin et al. 2001). The primary two types are glucose-excited (GE neuron, i.e. classic GR neuron) and glucose-inhibited (GI neuron, i.e. classic GS neuron), which are inhibited or excited when extracellular glucose levels decrease from 2.5 mM or 5 mM to 0.1 mM. In addition, they identified three types of presynaptic glucose sensing neurons, which change firing status by presynaptic inputs coming from other glucose sensing neurons. These neurons are: PDE neurons that can be presynatically excited by decreased glucose levels, PER neurons that can be presynaptically excited by increased glucose levels, and PIR neurons that can be presynaptically inhibited by increased glucose levels.

Glucose sensing neurons have also been identified in the hindbrain AP/NTS area (Mizuno and Oomura 1984; Riediger, Schmid et al. 2002). Twenty-six percent of the NTS neurons examined in the brainstem slices responds to changes in glucose levels; of which 76% decrease neuronal activity (GS neuron) and 24% increase neuronal activity (GR neuron) when glucose levels increase from 3 mM to 10 mM. The caudal part of NTS contains more glucose-responding neurons than the rostral part of NTS (Mizuno and Oomura 1984). In the AP, 53% of the neurons respond to changes in glucose levels, and all of them decrease activity at decreased glucose levels (GR neuron) (Riediger, Schmid et al. 2002). Taken together, hypothalamus and AP/NTS contain glucose sensing neurons that alter neuronal activity in response to fluctuation of blood glucose levels.

The Role of Glucose Metabolism in Neuronal Glucose Sensing

The role of glucose metabolism in the mechanism of neuronal glucose sensing has been examined in the glucose responsive (GR) neurons in the VMH (Yang, Kow et al. 1999). GR neurons in the VMH increase firing when glucose is changed from 5 mM to 20 mM, but not from 1 mM to 5 mM. The stimulatory effect of glucose on the VMH GR neurons is blocked by simultaneous addition of 2DG, glucokinase inhibitor glucosamine, glucose transporter inhibitor phloridzin, and glyceraldehyde phosphate dehydrogenase (GAPDH) inhibitor iodoacetic acid. On the other hand, glucose effect on GR neurons can be mimicked by glycolytic intermediate glyceraldehyde and lactate, but not pyruvate. These results suggest that glucose metabolism is necessary for stimulation of GR neuron activity. Sulphonylurea tolbutamide can also stimulate GR neurons, indicating the existence of ATP-sensitive K channel. Despite some differences, VMH GR neurons exhibit a great similarity to the pancreatic β cells in terms of glucose sensing, such as requirement for glucose metabolism and the presence of K_{ATP} channel.

The role of glucose metabolism in the inhibitory effect on neuronal activity is also examined in the GS neurons in the VMH (Yang, Kow et al. 2004). VMH GS neurons are inhibited when glucose is changed from 5 mM to 20 mM. Inhibitors of glucose transport (phloridzin), glucokinase (glucosamine), or glycolytic enzyme GAPDH (iodoacetic acid) block the inhibitory effect of glucose on GS neurons. Glycolytic intermediates glyceraldehyde and lactate, but not pyruvate, can mimic the inhibitory effect of glucose on GS neurons. 2DG blocks the inhibitory effect of glucose on GS neurons, and even activates GS neurons in the presence of 20 mM glucose. Unlike GR neurons, tolbutamide has no effect on GS neurons, indicating that the K_{ATP} channel is not necessary for glucose inhibition of GS

neurons. Taken together, glucose metabolism is required for the inhibitory effect of glucose on GS neurons in the VMH.

Hypothalamic Glucose Sensing Model

As mentioned above, glucose sensing neurons, especially GR neurons, exhibit a great similarity to pancreatic β cells. In addition, the β cell glucose sensing components, GLUT2, βGK, and K_{ATP} channel have been identified in the hypothalamus (Navarro, Rodriquez de Fonseca et al. 1996; Yang, Kow et al. 1999; Schuit, Huypens et al. 2001). The highest expression of GK in the brain is in the arcuate nucleus and VMH (Lynch, Tompkins et al. 2000). A model for glucose sensing in the hypothalamus has been proposed based on the β cell model (Schuit, Huypens et al. 2001). In this model, glucose metabolism through glycolysis is necessary for signaling neuronal activity. As in the β cells, GK is likely rate limiting in the glucose flux through glycolysis, leading to proportional generation of ATP. Increased ATP/ADP ratio results in closure of K_{ATP} channels and triggers firing of GR neurons. In addition to glucose, the hypothalamic glucose sensing neurons may interact with endocrine signals from insulin and leptin to regulate energy intake and energy expenditure (Spanswick, Smith et al. 1997; Spanswick, Smith et al. 2000).

The Pancreatic β Cell Glucose Sensing and Insulin Secretion Model

The pancreas is an important peripheral glucose-sensing organ. Pancreatic β cells can detect fluctuation of blood glucose level and respond by regulation of insulin/glucagon secretion. Previous findings have demonstrated that glucokinase (GK), glucose transporter 2 (GLUT2) and ATP-sensitive potassium (K_{ATP}) channels are required for normal glucose sensing and insulin secretion in pancreatic β cells. GLUT2 (Km = ~ 17 mM for glucose) plays a permissive role and allows rapid glucose entry into pancreatic β cells (Thorens 2001). GK is the rate-limiting enzyme of glycolysis and regulates the rate of glucose metabolism in

β cells (Matschinsky, Liang et al. 1993). Glucose enters the β cells via GLUT2 and is metabolized through glycolysis, resulting in the generation of ATP. Increased intracellular ATP closes ATP-sensitive potassium channels on the membrane of β cells, and the resultant decrease in the efflux of potassium leads to depolarization of the β cells, opening of voltage-sensitive calcium channels, influx of calcium and secretion of insulin (Kieffer and Habener 2000). In addition to glucose metabolism that can induce insulin secretion, other factors such as glucagon-like peptide-1 (GLP-1) can also induce insulin secretion (Drucker 1998; Drucker 2001). The components of β cell glucose sensing apparatus, including GLUT2, βGK, K_{ATP} channel, have been detected in specific brain areas involved in the regulation of food intake (Leloup, Arluison et al. 1994; Karschin, Ecke et al. 1997; Lynch, Tompkins et al. 2000). And it has been proposed that brain glucose sensing neurons use a similar mechanism like β cell glucose sensing model (Schuit, Huypens et al. 2001). Next, GLUT2, βGK, K_{ATP} channel, GLP-1 and GLP-1 receptor will be reviewed individually on their involvement in the β cell glucose sensing, their expression in the brain and proposed role in the brain glucose sensing and regulation of food intake, with an emphasis on GLUT2 and GK.

Glucokinase (GK)

GK Overview

GK, also referred to as type IV kexokinase, converts glucose to glucose-6-phosphate as the first step of glycolysis. Unlike hexokinase I, II, and III, GK has a high Km (~ 10 mM) for glucose and lacks the feedback inhibition by end product glucose-6-phosphate. Peripherally, it has been long established that GK is expressed in the liver and pancreatic islets β cells (Andreone, Printz et al. 1989; Magnuson, Andreone et al. 1989; Magnuson and Shelton 1989). In addition, GK is also identified in the pancreatic islet α cells (Heimberg, De Vos et al. 1996) and intestinal endocrine cells (Jetton, Liang et al. 1994). Liver GK (LGK)

and β cell GK (βGK) are transcribed under different promoters, resulting in tissue-specific GK isoforms in the liver and β cells, which differ in their sequence of N-terminal amino acids (Magnuson and Shelton 1989). Pancreatic islet α cell GK mRNA and protein are same as βGK (Heimberg, De Vos et al. 1996). In the gut, GK transcription is under the same promoter as βGK (Jetton, Liang et al. 1994).

Liver GK

Liver GK is responsible to quickly phosphorylate glucose and direct glucose to glycogen synthesis and lipogenesis after a meal. In the liver, glucose metabolism regulates the expression of glycolytic and lipogenic enzymes, such as pyruvate kinase and fatty acid synthase in a dose-dependent manner. LGK plays a key role in regulating liver glucose metabolism, and is required for glucose induction of glycolytic and lipogenic enzymes (Girard, Ferre et al. 1997). Consistently, fasted rats with GK overexpression in the liver show increased pyruvate kinase activity similar to control fed rats. This also demonstrates that GK is the rate-limiting step in liver glucose utilization and glucose-regulated glycolytic enzyme expression (Ferre, Riu et al. 1996; Scott, Collier et al. 2003). Liver specific GK knock out does not affect animal survival. The LGK knock out mice show a 10% increase in plasma glucose and doubled plasma insulin in the fed state, indicating insulin resistance. During hyperglycemic clamp, these mice show 30% of glucose turnover and 10% of liver glycogen synthesis ability compared with control mice, suggesting impaired liver glucose disposal (Postic, Shiota et al. 1999). The above evidence indicates that LGK plays an important role in liver glucose metabolism and in the whole body glucose homeostasis.

Insulinoma-bearing rats develops hyperinsulinemia and hypoglycemia after 2-3 months of insulinoma implantation. In these rats, LGK activity is 4-fold that of control. Glucose infusion for 24 h brings glucose levels back to 10 mM, but has no effect on LGK

activity. Removal of insulinoma brings glucose and insulin back to normal level, LGK activity decreases to control level by day 6 after insulinoma removal (Bedoya, Matschinsky et al. 1986). LGK mRNA increases 20-fold and 30-fold in diabetic rats after infusion of insulin for 2 and 4 h, respectively (Magnuson, Andreone et al. 1989; Magnuson and Shelton 1989). In 2-day fasted rats, a single dose of glybenclamide (0.1 mg / kg body weight) (close K-ATP channel and induce insulin secretion) induces a 4-fold increase in LGK mRNA within 1 h; the induction of LGK mRNA is abolished by concomitant administration of diazoxide (open K-ATP channel and antagonize insulin secretion) with glybenclamide (Tiedge and Lenzen 1995). The above evidence suggests that LGK is regulated at the transcriptional level by insulin but not glucose.

Regulatory Protein of Glucokinase (GKRP)

Liver GK is also regulated by glucokinase regulatory protein (GKRP). GKRP is mainly located in the nucleus of the hepatocytes. At normal glucose concentrations (5 mM), GK binds to GKRP and is retained in the nucleus; at high glucose concentrations (10-30 mM) or in the presence of low concentrations of fructose (50 uM to 1 mM), GK is released and translocates into the cytoplasma for glucose phosphorylation (Agius and Peak 1993; Van Schaftingen 1994; Van Schaftingen, Detheux et al. 1994; Agius, Peak et al. 1995; Brown, Kalinowski et al. 1997). Binding of GKRP to GK inhibits GK activity. Adenovirus-mediated overexprsssion of GKRP in the isolated hepatocytes increases bound GK and decreased free GK. GKRP overexpression inhibits free GK activity, glucose phosphorylation, glycolysis and glycogen synthesis (de la Iglesia, Mukhtar et al. 2000). The effect of GKRP deficiency is also investigated by using GKRP knockout mice. GKRP deficient mice show absence of GK anchored to the nucleus in the liver cells (Farrelly, Brown et al. 1999; Grimsby, Coffey et al. 2000). Compared with wild-type mice, the homozygous ($GKRP^{-/-}$) and heterozygous

($GKRP^{+/-}$) GKRP knockout mice have similar basal blood glucose and insulin levels. After a glucose load, the $GKRP^{-/-}$ mice show moderately impaired glucose tolerance. At 5 mM glucose, GK activity in the liver homogenates from $GKRP^{-/-}$ and $GKRP^{+/-}$ mice is not different from controls. At 50 mM glucose, GK protein is decreased by 75% and 30%; whereas GK mRNA is increased by 1.7- and 2.1-fold in $GKRP^{-/-}$ and $GKRP^{+/-}$ mice, compared with control. The decrease in GK protein is not due to decreased GK transcription. GKRP knockout study suggests that GKRP binding to GK serves as a "functional reserve" for GK to be activated by metabolic milieu, and GKRP may also helps stabilize live GK protein (Grimsby, Coffey et al. 2000). Consistently, another GKRP-mutant study shows that GK protein and activity are decreased in the liver of GKRP-mutant mice. The GKRP-mutant mice also show impaired glucose tolerance. In the GKRP-mutant mouse liver, insulin stimulates GK mRNA but fails to increase GK protein, supporting that GKRP regulates liver GK expression at the posttranscriptional level (Farrelly, Brown et al. 1999). In support that GKRP may help stabilize GK, adenovirus-mediated GKRP and GK are overexpressed in the HepG2 cells, which do not express endogenous GK and GKRP. Cells with both GKRP and GK overexpression have higher GK protein and enzymatic activity than cells with only GK overexpression, suggesting that GKRP may help to stabilize or protect GK (Slosberg, Desai et al. 2001). Taken together, GKRP binds to and localize liver GK in the nucleus in an inactive status. The GKRP-GK complex serves as a reservoir and releases GK in response to metabolic stimuli. GKRP also plays a regulatory role in the liver GK protein expression at the posttranscriptional or posttranslational level, and GKRP may help stabilize GK as well.

GKRP mRNA and protein are also identified in the pancreatic islets and hypothalamus (Alvarez, Roncero et al. 2002). In addition to liver isoform of GKRP, two alternative splicing isoforms of GKRP are also found in the liver, pancreatic islets and

hypothalamus. Like in the liver, brain GKRP can be co-precipitated with GK, indicating that GKRP interacts with GK in the brain. In the brain, GKRP and GK are mainly in the soluble and nuclear fractions, similar as that of liver. However, whether GKRP in the pancreatic islet and in the hypothalamus play a regulatory role on GK as in the liver is still unknown.

GKRP overexpression in the liver may be used as a tool to treat type II diabetes. In a type II diabetic mouse model, GKRP overexpresssion in the liver is achieved by injection of adenovirus containing human GKRP through tail vein. Three weeks after injection, the diabetic mice with GKRP overexpression in the liver show lowered blood glucose similar to that of non-diabetic control mice, as well as improved glucose tolerance, implicating the possibility of GKRP as a treatment for type II diabetes (Slosberg, Desai et al. 2001).

β Cell GK

βGK has been known as the pancreatic β cell glucose sensor (Matschinsky, Liang et al. 1993; Postic, Shiota et al. 2001). βGK phosphorylation of glucose is the rate-limiting step in glycolysis in the β cells, and βGK determines the rate of glucose metabolism in the β cells, which proportionally reflects blood glucose levels. In this way, βGK acts as a glucose sensor and couples glucose metabolism to insulin secretion.

βGK has also been proposed as a diabetes candidate gene (Matschinsky, Liang et al. 1993; Postic, Shiota et al. 2001). Mutations in GK have been identified as the cause of MODY2 (maturity onset diabetes of the young) in humans. MODY2 is autosomal dominant, familial inherited and has mutations in one allele of GK gene. Patients with MODY2 are characterized by early age onset of type II diabetes, impaired GK enzymatic activity, and a rightward shift of the glucose stimulated insulin secretion curve (Velho and Froguel 1998). Animal model for MODY has been generated by disruption of exon 2 of GK gene (Bali, Svetlanov et al. 1995). Heterozygous mice with GK mutations ($GK^{+/-}$) show similar

characteristics like that of MODY, including reduced both βGK and LGK enzymatic activity, mildly elevated fasting glucose levels, and impaired glucose tolerance as revealed by hyperglycemic clamp. To specify the role of βGK, βGK knockout mice has been generated by disruption of exon 1β of GK gene, and liver GK is intact in these mice (Terauchi, Sakura et al. 1995). The homozygous ($GK^{-/-}$) mice die within seven days after birth due to severe diabetes. The heterozygous ($GK^{+/-}$) mice show mild glycosuria within a day after birth suggesting early onset, and significant increase in blood glucose levels and impaired glucose tolerance at ten weeks of age, suggesting diabetes. This study indicates that homozygous βGK mutation is lethal, and heterozygous βGK mutation is sufficient to cause diabetes like MODY. Postic et al (Postic and Magnuson 1999) have produced another β cell GK specific knock out mouse model by using Cre-LoxP system. Consistently, most of the homozygous βGK knockout mice ($\beta GK^{-/-}$) die of severe diabetes within a week after birth; and the heterozygous βGK knockout mice ($\beta GK^{+/-}$) can survive. In contrast, both homozygous and heterozygous liver GK knockout mice survive. At six to ten weeks of age, the $\beta GK^{+/-}$ mice are moderately hyperglycemic, and demonstrate reduced insulin secretion (by 70%), glucose turnover (by 60%) and glucose infusion rate (by 70%) during hyperglycemic clamp. Taken together, βGK plays an indispensable role in the whole body glucose homeostasis.

βGK is differentially regulated from liver GK. An insulinoma-bearing rat model has been generated by subcutaneous implantation of a small fragment of insulinoma (Bedoya, Matschinsky et al. 1986). Two to three months later, the insulinoma-bearing rats show hyperinsulinonemia and hypoglycemia, and βGK activity is only about 30% in the islets compared with control. After 24 h 50% glucose infusion, βGK activity doubles and reaches 50% of controls. βGK activity is fully recovered to control level 24 h after removal the insulinoma when hypoglycemia has been corrected. This study suggests that glucose plays a

primary role in the regulation of βGK. In two-day fasted rats, oral glucose (4 g / kg body weight) induces βGK mRNA three-fold within 1 h (Tiedge and Lenzen 1995). βGK mRNA in the isolated rat islets incubated in 10 mM glucose increases to 2.8-fold of that incubated in 2 mM glucose. This evidence further supports that glucose regulates βGK gene expression.

Glucose also regulates enzymatic activity of βGK at the post-translation level. Pancreatic islets cultured in 10 mM glucose show 116% increase in βGK enzyme activity compared with 2 mM glucose (Tiedge, Steffeck et al. 1999). Evidence for whether glucose induces βGK translocation does not agree very well. Noma et al show that glucose infusion (1 g / kg body weight) rapidly induces a translocation of βGK from a perinuclear position into the cytoplasm in rat islets (Noma, Bonner-Weir et al. 1996). By using GK overexpressing insulin-secreting RINm5F cells, Tiedge et al show that GK exists in two fractions: a bound fraction with low enzyme activity and a diffusible fraction with high activity; and glucose induces a significant increase in the diffusible GK fraction with high enzyme activity. A regulatory protein that may regulate βGK activity in response to glucose has been suggested, and this protein is not identical to liver GKRP as revealed by Northern Blot (Tiedge, Steffeck et al. 1999). In another study, a glucose-responsive insulin secreting β cells line, MIN6 cells are used to examine the post-translation regulation of glucose on GK. In the MIN6 cells, GK exists in the cytoplasm as well as in granules; 25 mM glucose fails to induce a rapid change in the subcelluar localization of GK compared with 5 mM glucose, and therefore argues against the existence of liver GKRP like regulatory protein (Stubbs, Aiston et al. 2000). On the other hand, both mRNA and protein of GKRP have been reported to exist in the pancreatic islets, and this GKRP can interact with GK (Alvarez, Roncero et al. 2002). In summary, glucose stimulates βGK enzyme activity. It is still inconclusive whether glucose

induces translocation and release of GK in an active status, and whether βGK is regulated by a liver GKRP-like protein in a glucose-dependent manner.

Small Molecule GK Activators

A small molecule GK activator (GKA) has been synthesized based on the screening of a library of 120,000 synthetic compounds. GKA dose-dependently increases GK activity and reverses the inhibition of GK by GKRP. GKA lowers the threshold concentration of glucose to induce insulin secretion in freshly isolated rat pancreatic islets (from 7 mM to 3 mM). A single oral dose of GKA lowers blood glucose in wild type and type II diabetic C57BL/6J mice. The decrease in blood glucose is accompanied with an increase in blood insulin in the wild type mice. Glucose tolerance test shows that GKA improves glucose tolerance in ob/ob and diet-induced obese mice. GKA also exerts a favorable effect on liver by increasing hepatic glucose uptake and inhibiting endogenous glucose production (Grimsby, Sarabu et al. 2003). The above evidence suggests that GKA can be a potential anti-hyperglycemia and anti-diabetic drug.

GK in the Brain

Jetton et al first analyzed the upstream GK promoter activity in the transgenic mouse brain. The upstream GK promoter is the β cell GK promoter. In the brain of the transgenic mice, the upstream GK promoter activity is detected in the pituitary and medial hypothalamus, RNA-PCR showed GK mRNA in the hypothalamus, cortex, brainstem and cerebellum (Jetton, Liang et al. 1994). This study demonstrates the existence of GK in the brain, and it is the βGK promoter that is active in the brain. Later on, two studies show that βGK but not liver GK is the predominant isoform of GK expressed in the hypothalamus by RT-PCR (Yang, Kow et al. 1999; Schuit, Huypens et al. 2001). Lynch and colleagues (Lynch, Tompkins et al. 2000) demonstrate the relative abundance of GK mRNA in the

hypothalamus and in the hindbrain by in situ hybridization and RT-PCR. In situ hybridization shows that the heaviest labeling for GK is in the arcuate nucleus and the ventromedial nucleus within the hypothalamus. Double labeling shows that more than 75% of NPY neurons in the arcuate nucleus co-express GK mRNA. RT-PCR shows the highest GK levels in the arcuate nucleus and the lateral hypothalamus and the lowest in the paraventricular nucleus. A more recent study (Dunn-Meynell, Routh et al. 2002) reports the presence of GK mRNA in AP/NTS by RT-PCR; furthermore, GK has been localized specifically in the neurons by double-labeling in situ hybridization. In summary, GK expression is "neuron-specific" in the discrete hypothalamic and hindbrain areas; and βGK is the predominant isoform of GK in the brain.

GK has been proposed as the likely "glucose sensor" in hypothalamic glucose-excited (GE) and glucose-inhibitory (GI) neurons (Dunn-Meynell, Routh et al. 2002). GE and GI neurons have been isolated from ventromedial hypothalamic nucleus. At 2.5 mM glucose, GE neurons are stimulated and GI neurons are inhibited; at 0.5 mM glucose, GE neurons are inhibited and GI neurons are stimulated. At 2.5 mM glucose, addition of selective GK inhibitors (alloxan, mannoheptulose, glucosamine or N-acetylglucosamine) result in inhibition of GE neurons and stimulation of GI neurons, suggesting that GK acts as a glucose sensor in these neurons. Injection of alloxan into the third ventricle, but not fourth ventricle, results in reduced food intake and hyperglycemic response to 2DG or 5TG. The destruction of tanyacytes and neuronal swelling after alloxan injection may be responsible for the above feeding and glycemic response. Upon the recovery of the tanyacytes, the feeding and glycemic response to glucoprivation are restored (Sanders, Dunn-Meynell et al. 2004). The conclusion from this report is that "neuronal substrates around third ventricle affected by alloxan" is responsible for the above response. However, it is also possible that some changes

have been induced by alloxan in the tanyacytes that result in the subsequent neuronal changes and behavioral changes. Alloxan is not a specific GK inhibitor; it may affect factors unknown at present. GLUT2 has been shown expressed in the tanyacytes along the third ventricle, and the tanyacytes in the median eminence (which is close to the bottom of the third ventricle) have direct contact with the neurons in the arcuate nucleus (Garcia, Millan et al. 2003). Therefore, the possibility of GLUT2 involvement in this response cannot be excluded.

Glucose Transporter 2 (GLUT2)

GLUT2 Overview

Glucose transporter 2 (GLUT2) is a low affinity and high Km (~ 17 mM) facilitative glucose transporter, and GLUT2 transport of glucose is bi-directional. GLUT2 protein contains 522 amino acids; the predicted structure of GLUT2 consists of 12 membrane-spanning domains with both the N-terminal and C-terminal in the cytoplasm. There is a single glycosylation site between the first and second membrane-spanning domain, and there is a large intracellular loop located between the membrane-spanning domains six and seven (Thorens, Sarkar et al. 1988; Olson and Pessin 1996).

Peripherally, GLUT2 is expressed in the liver, pancreatic β cells, small intestine and kidney ((Thorens, Sarkar et al. 1988), tissues that require the high Km GLUT2 to rapidly transport glucose in proportion to physiological glucose concentrations. Unlike GK, GLUT2 is not detected in the pancreatic α cells (Heimberg, De Vos et al. 1995).

Liver GLUT2

Thorens et al (Thorens, Flier et al. 1990) show that GLUT2 is the major glucose transporter in the liver. Liver GLUT2 protein is not changed after a two-day fasting and is increased 70% after refeeding. In contrast to the minimal changes in the protein, liver

GLUT2 mRNA is decreased 45% after two-day fasting and increased up to five fold after refeeding, compared with ad libitum controls. Liver GLUT2 protein and mRNA are not altered in streptozotocin-induced diabetic rats, either with or without insulin treatment. Compared with the more dramatic changes in liver GLUT2 mRNA by fasting and refeeding, GLUT2 protein changes within a small range. The rather constant level of GLUT2 protein is consistent with the role of GLUT2 in the liver as a bi-directional transporter for glucose. After feeding, GLUT2 transports glucose into the liver cells for metabolism, and during fasting period, GLUT2 transports glucose derived from glycogenolysis and gluconeogenensis out of liver cells to maintain blood glucose levels.

The regulation of liver GLUT2 by metabolic alterations has also been investigated in isolated rat hepatocytes (Asano, Katagiri et al. 1992; Rencurel, Waeber et al. 1996). Glucose upregulates GLUT2 mRNA in a dose dependent manner in the hepatocytes; and there is a two-fold increase in GLUT2 mRNA at 27.8 mM glucose compared with no glucose. Fructose and mannose also upregulate GLUT2 mRNA in the hepatocytes (Asano, Katagiri et al. 1992). The up-regulation of liver GLUT2 by glucose requires glucose metabolism and occurs at the transcriptional level (Rencurel, Waeber et al. 1996). Non-metabolizable glucose analog 3-*O*-methylglucose and 2-deoxyglucose cannot stimulate GLUT2 mRNA in primary hepatocytes. Glucose induction of GLUT2 mRNA is abolished in cultured hepatocytes after blocking of glucose phosphorylation; dihydroxyacetone can stimulate GLUT2 mRNA in hepatocytes, whereas pyruvate and lactate cannot, indicating that a metabolite from glucose metabolism is needed for glucose stimulation of GLUT2. Glucose regulates GLUT2 at the transcription level by activating the proximal region of GLUT2 promoter. Taken together, liver GLUT2 is induced by glucose, which requires glucose metabolism, and the induction by

glucose involves regulation at transcription of GLUT2 mRNA by activation of GLUT2 promoter.

β Cell GLUT2

GLUT2 is a glucose sensor in the pancreatic β cells. GLUT2 plays a permissive role in the β cells, allowing the uptake of glucose by β cells to be proportional to blood glucose, which is important because signals generated from glucose metabolism to regulate insulin secretion in the β cells has to reflect glucose fluctuation in the blood. In this sense, GLUT2 is essential for glucose stimulation of insulin secretion (GSIS).

It had been proposed that defective glucose transporter in the β cells results in loss of GSIS, hyperglycemia, and noninsulin-dependent diabetes (NIDDM) (Johnson, Ogawa et al. 1990; Unger 1991). This hypothesis is tested in a NIDDM animal model, Zucker diabetic fatty rats. In the diabetic Zucker rats, hyperglycemia (blood glucose > 11 mM) is associated with loss of GSIS, reduced glucose transport and decreased GLUT2 in the β cells. There is a negative relationship between hyperglycemia and the percentage of GLUT2-positive β cells; and GSIS is absent when the percentage of GLUT2-positive β cells is less than 60%. In addition, down-regulation of GLUT2 is not secondary to hyperglycemia. Hyperglycemia maintained for seven days in normal rats by continuous infusion of 50% glucose fails to down-regulate GLUT2 in β cells. In vitro, neonatal rat pancreas cultured in the presence of high glucose (33.4 mM) for five weeks even show increased GLUT2 protein. Prevention of hyperglycemia with acarbose (inhibitor of α-glucosidase) does not prevent the loss of GLUT2 in prediabetic Zucker rats (Orci, Ravazzola et al. 1990). Taken together, reduction of GLUT2 in the β cells results in defective glucose sensitivity and insulin response of β cells, and may play a role in the development of persistent hyperglycemia and NIDDM.

The role of GLUT2 in the β cells is further investigated using GLUT2-null mice (Guillam, Hummler et al. 1997). The homozygous GLUT2-null mice have abnormal pancreatic islets development (inversion of α and β cell ratio), hyperglycemia, hypoinsulinemia, and impaired glucose tolerance (persistent hyperglycemia 90 min after glucose load). The GLUT2-negative islets show delayed and decreased insulin secretion to 16.7 mM glucose. Glucose-stimulated insulin biosynthesis is also impaired in the GLUT2-negative islets (Guillam, Hummler et al. 1997). Lentivirus-mediated GLUT2 re-expression in the GUT2-null islets restores the normal insulin secretion (Guillam, Dupraz et al. 2000). In another report (Thorens, Guillam et al. 2000), the GLUT2-null mice are re-expressed GLUT1 or GLUT2 in pancreatic β cells by transgenic approach. Re-expression of either GLUT1 or GLUT2 restores the glucose tolerance and insulin secretory response during hyperglycemic clamp. In vitro, islet isolated from GLUT2-null mice with either GLUT1 or GLUT2 re-expression show normal GSIS when treated with 16.7 mM glucose, in which the first phase of insulin secretion and the amplitude of insulin secretion are restored to control levels. One of the conclusions from this study is that it is the rate of glucose uptake at high glucose concentration that is important for normal GSIS. However, under physiological conditions, only GLUT2 has the high Km (~17 mM) that allows glucose transport into the β cells in proportion to blood glucose levels and in an unsaturated manner. The kinetic property of GLUT2 confers it as a glucose sensor for β cells.

In contrary to the above study, an in vitro study using AtT-20ins cells shows that it is GLUT2, but not GLUT1 overexpression that enables the cells to secrete insulin in response to glucose stimulation (Hughes, Quaade et al. 1993). AtT-20ins cell line is derived from anterior pituitary and engineered to express human insulin. AtT-20ins cells overexpressing GLUT2 by transfection acquire the property of glucose stimulated insulin secretion, whereas

cells transfected with GLUT1 do not. Further examination shows that cells transfected with GLUT2 have a Km of 16-17 mM for 3-*O*-methyl glucose uptake, whereas cells transfected with GLUT1 have a Km of 4 mM, although glucose utilization is similar in both cells. The GLUT1 transfected cells do not reach a Km comparable to that of GLUT2, which explains the absence of GSIS, and emphasizes that it is the glucose uptake rate that is important in GSIS. On the other hand, the similar glucose utilization of both transfected cells suggestes that GSIS in the AtT-20 cells is not related to glucose metabolism, which raises the speculation that GLUT2 may signal to insulin secretion by other mechanisms in addition to glucose transport and the subsequent glucose metabolism in the β cells (Hughes, Quaade et al. 1993). This speculation is supported by the study with an engineered human hepatoma cell line. The hepatoma cells lack endogenous GLUT2 expression. After first transfection with insulin cDNA, the cells can synthesis, storage and release insulin to insulin secretagogues but not to glucose. After a second transfection with GLUT2 cDNA, the cells demonstrate GSIS. Surprisingly, ATP-sensitive potassium channel is induced in the insulin and GLUT2 double transfected cells (Liu, Simpson et al. 2003). Studies with pancreatic β cells suggeste that phosphorylation of the intracellular carboxyl terminal of GLUT2 protein may be involved in the stimulation of insulin secretion (Thorens, Deriaz et al. 1996). In summary, GLUT2 acts as a glucose sensor in β cells through glucose transport function as well as other mechanisms unknown at present.

GLUT2 in the β cells is regulated by glucose. GLUT2 mRNA of β cells is reduced 23%, 85%, and nearly 100% after 3-h, 4-day and 12-day hypoglycemia induced by insulin. Consistently, the islets from the rats after 12-day hypoglycemia lose the high Km for 3-O-methylglucose transport, which decreases from 17-20 mM to 2.5 mM. After 5-day hyperglycemia induced by continuous glucose infusion, islet GLUT2 mRNA is increased

46% (Thorens, Deriaz et al. 1996). In vitro study (Ferrer, Gomis et al. 1993) shows that GLUT2 mRNA from islets cultured in 11 or 16.7 mM glucose is 10-fold of that from islets cultured in 2 mM glucose. Correspondingly, 16 mM glucose induces a 4-fold increase in islet GLUT2 protein. Addition of RNA and protein inhibitors prevent glucose stimulation of islet GLUT2, indicating that the effect of glucose on GLUT2 involves mRNA and protein synthesis. In the presence of glycolysis inhibitor mannoheptulose, 16 mM glucose fails to induce GLUT2 mRNA and protein; 2-deoxyglucose cannot stimulate GLUT2 expression, suggesting that glucose metabolism is required for its stimulatory effect on islet GLUT2. Altogether, β cell GLUT2 is regulated by glucose.

GLUT2 in the Brain

Leloup and colloegues (Leloup, Arluison et al. 1994) demonstrat that relatively high levels of GLUT2 mRNA are expressed in the nucleus of the solitary tract (NTS) and the motor nucleus of the vagus (MNV) by reverse transcription-polymerase chain reaction (RT-PCR). In other brain areas, including the lateral hypothalamus, the arcuate nucleus, the olfactory bulb and the paraventricular nucleus, GLUT2 mRNA is merely detectable. Immunohistochemistry confirms the existence of GLUT2 in the above brain areas, with high immunoreactivity in the NTS-MNV and arcute nucleus. Further confirmation by Western Blot fails to show GLUT2 protein in the arcuate nucleus and NTS, suggesting the very low level of GLUT2 protein in these areas. Electronic microscope examination indicates that GLUT2 is expressed in the astrocytes but not neurons. Brant and colleagues (Brant, Jess et al. 1993) show by Western Blot that GLUT2 is expressed at all regions of the brain including the hypothalamus; and the expression level is too low to be quantified. A more recent study reports GLUT2 protein in the tanycytes along the third ventricle in the hypothalamus (Garcia, Millan et al. 2003). Tanycytes are highly elongated ependymal cells, and there are four types,

α1, α2, β1, and β2. α1 and α2 tanycytes line the dorsal walls of the third ventricle and mainly project to the ventromedial nucleus of the hypothalamus. β1 tanycytes line the lower part of the third ventricle and may contact the arcuate nucleus. β2 tanycytes line the floor of the third ventricle and contact the median eminence. The presence of GLUT2 in the tanycytes indicates that GLUT2 may detect glucose concentration in the blood and the cerebrospinal fluid and send signals to neurons in the ventromedial nucleus and arcuate nucleus. Thus GLUT2 may play a role in the brain glucose sensing, like in the β cells. Taken together, GLUT2 is expressed in the brain at low levels and the localization of GLUT2 suggests that it may play a role in the brain glucose sensing.

Wan and colleagues (Wan, Hulsey et al. 1998) show that ICV injection of antisense oligos against GLUT2 mRNA in rats causes significant suppression in food intake by the 12th hour after injection, and remained suppressed at 24 h. The cumulative food intake is significantly lower in the antisense-injected rats. They also show that in antisense-treated rats, 2DG fails to stimulate feeding compared with vehicle control, whereas in missense treated rats, 2DG significantly increases food intake. Leloup and colleagues (Leloup, Orosco et al. 1998) injected antisense oligos to GLUT2 directly into arcuate nucleus twice daily for two days. After the first day injection, the rats administered antisense oligos have a significant body weight loss compared with missense and saline injected rats, which is not due to a decreased food intake, suggesting that GLUT2 may be involved in regulation of energy expenditure. Antisense treatment abolishes hyperinsulinemia induced by carotid artery injection of a small glucose load, indicating that GLUT2 may play a role in the brain glucose sensing. In summary, GLUT2 may be involved in the brain glucose sensing, regulation of food intake, and energy expenditure.

ATP Sensitive K (K_{ATP}) Channel

In the Pancreas

Like GLUT2 and GK, K_{ATP} channel is required for normal glucose sensing and insulin secretion of pancreatic β cells (Ashcroft and Gribble 1998). The β cells K_{ATP} channels consist of two subunits: a sulphonylurea receptor subunit (SUR1) and an inwardly rectifying K-channel subunit (Kir6.2). SUR1 is the regulatory subunit of K_{ATP} channels; and Kir6.2 is the pore-forming subunit. Sulphonylureas and diazoxide binding to SUR1 result in inhibition and activation of K_{ATP} channels. ATP probably binds to Kir6.2 and inhibits K_{ATP} channels; and MgADP binds to SUR1 and activates K_{ATP} channels. Both kir6.2 and SUR1 are important in the regulation of K_{ATP} channels activity and insulin secretion.

In the Brain

K_{ATP} channel components, Kir6.2 and SUR1, are widely expressed in the brain, including cortex, hippocampus, midbrain, hypothalamus, cerebellum and hindbrain (Karschin, Ecke et al. 1997; Dunn-Meynell, Rawson et al. 1998). Kir6.2 is primarily located in neurons, but not glial cells (Dunn-Meynell, Rawson et al. 1998). The widespread expression of K_{ATP} channels suggests that K_{ATP} channels may play a vital role in the brain function, such as neuroprotection and silencing of neurons in the presence of extreme hypoglycemia (Mobbs, Kow et al. 2001). Further, in situ hybridization shows that in the hypothalamus and hindbrain, expression of Kir6.2 mRNA and SUR1 mRNA is overlapped (Karschin, Ecke et al. 1997), Kir6.2 mRNA is colocalized with NPY mRNA in the arcuate nucleus in the hypothalamus (Dunn-Meynell, Rawson et al. 1998). Single neuron RT-PCR shows that Kir6.2 and SUR1 mRNA was coexpressed in the GABA neurons of ventromedial hypothalamus (VMH), Taken together, the pancreatic β cell K_{ATP} channel is widely expressed in the brain.

In addition to global neuroprotection in the presence of extreme hypoglycemia, the role of K_{ATP} channel in the whole body glucose homeostasis has been examined in the Kir6.2-deficient (Kir6.2$^{-/-}$) mice. The Kir6.2$^{-/-}$ mice cannot increase glucagon secretion after systemic hypoglycemia induced by insulin injection, although glucagon secretion in response to low glucose (1 mM) in cultured islets from Kir6.2$^{-/-}$ mice is similar to control. The Kir6.2$^{-/-}$ mice cannot increase glucagon secretion in response to third ventricle injection of 2-deoxyglucose, whereas the control mice can. The impaired counter-regulatory response to hypoglycemia and central glucoprivation suggests that central K_{ATP} channel play a role in the whole body glucose homeostasis (Miki, Liss et al. 2001). VMH neurons from Kir6.2$^{-/-}$ mice display a higher spontaneous activity at 2.5 mM glucose but do not increase firing when changed to 25 mM glucose, compared with control, implying that VMH neurons require Kir6.2-containing K_{ATP} channel for glucose sensing. Because VMH is involved in the glucagon secretion (Borg, During et al. 1994; Borg, Sherwin et al. 1995; Borg, Sherwin et al. 1997), the abnormal glucose sensing of Kir6.2$^{-/-}$ VMH neurons results in the impaired glucagon response to hypoglycemia and glucoprivation.

K_{ATP} channel in the hypothalamic neurons is affected by insulin and leptin (Spanswick, Smith et al. 1997; Spanswick, Smith et al. 2000). In the normal Sprague-Dawley rats and lean Zucker rats, both leptin and insulin cause hyperpolarization and decreased firing of hypothalamic neurons by activation of K_{ATP} channel, which effect is reversed by sulphonylurea tolbutamide. In contrast, leptin and insulin have no effect on the firing rate of hypothalamic neurons from obese Zucker rats, implying that K_{ATP} channel plays a role in the central action of leptin and insulin, and therefore is involved in the central regulation of energy homeostasis. In summary, K_{ATP} channel in the brain is involved in global neuroprotection, central glucose sensing and regulation of glucose and energy homeostasis.

GLP-1 and GLP-1 Receptor

Function in Glycemic Control

Peripherally, enteroendocrine cells in the small intestine secrete GLP-1. GLP-1 receptors are G-protein coupled receptors; GLP-1 receptors have been localized on the membrane of pancreatic α and β cells. GLP-1 binds to GLP-1 receptor, activates Gs and adenylate cyclase (AC), increases cAMP levels and activates protein kinase A (PKA) and other downstream effectors, leading to insulin secretion. GLP-1 signaling not only stimulates glucose-dependent insulin secretion from β cells, but also activates insulin gene promoter and insulin transcription (Drucker 1998; Drucker 2001). GLP-1 and GLP-1 receptor play an important role in the regulation of insulin secretion and synthesis.

The study with GLP-1 receptor mutant ($GLP\text{-}1R^{-/-}$) mice demonstrates the importance of GLP-1 in glycemic control. $GLP\text{-}1R^{-/-}$ mice show increased fasting blood glucose, impaired glucose tolerance, irrespective of the route of glucose administration, and reduced insulin secretion 30 minutes after oral glucose challenge, compared with control (Scrocchi, Brown et al. 1996). Furthermore, $GLP\text{-}1R^{-/-}$ mice show normal fasting glucagon and suppression of glucagon after glucose challenge; a small but not significant decrease in fasting insulin mRNA in the islets, and normal glucose utilization in the whole body. Taken together, GLP-1 stimulation of insulin secretion plays an essential role in the GLP-1 effect on glycemic control (Scrocchi, Marshall et al. 1998).

In the Brain

Several studies have established the expression of GLP-1 and GLP-1R in the brain. GLP-1 mRNA expression is restricted to nucleus of the solitary tract (NTS) and the olfactory bulb. GLP-1R mRNA is detected in numerous brain regions including the hypothalamus and the hindbrain (Alvarez, Roncero et al. 1996; Merchenthaler, Lane et al. 1999). In situ

hybridization shows colocalization of GLP-1R and GLUT2 or GK mRNA in the ependymal cells along the third ventricle and the arcuate nucleus, median eminence and supraoptic nucleus (Navarro, Rodriquez de Fonseca et al. 1996).

GLP-1 is a mediator of satiety in the brain (Navarro, Rodriquez de Fonseca et al. 1996; Turton, O'Shea et al. 1996). Intracerebroventricular (ICV) injection of GLP-1 significantly reduces dark-phase food intake and food intake in 24 h fasting rats, compared with ICV saline control. ICV injection of GLP-1R antagonist, exendin (9-39), reverses GLP-1 inhibition on feeding. GLP-1 significantly attenuates and GLP-1R antagonist significantly potentiates NPY-induced feeding. ICV injection of GLP-1 induces c-fos expression in the paraventricular nucleus (PVN), and 80% of CRH neurons colocalize with c-fos expression. In addition, central GLP-1 also induces c-fos in the ventrolateral part of the arcuate nucleus (ARC), area postrema and nucleus of the solitary tract (AP/NTS) and amygdala (Turton, O'Shea et al. 1996; Larsen, Tang-Christensen et al. 1997). Altogether, central GLP-1 may exert its anorexic effect through activation of CRH neurons in the PVN, probably other anorexic neurons in other areas related to regulation of food intake. In contrast to the anorexic effect of acute administration of GLP-1 or GLP-1 receptor antagonist, GLP-1 receptor mutation in transgenic mice does not affect body weight and food intake compared with wild type mice, suggesting that GLP-1 mediated satiety is dispensable and can be compensated by other satiety mediators (Scrocchi, Brown et al. 1996). In summary, GLP-1 is one of the mediators of satiety and GLP-1 exerts its anorexic effect directly in the brain.

Summary

The hypothalamus plays an important role in the central regulation of energy homeostasis (Schwartz, Woods et al. 2000). Recently, AMPK has been implicated as the energy sensor in the hypothalamus and plays a central role in the regulation of food intake

(Minokoshi, Alquier et al. 2004). Glucose sensing in the hypothalamus contributes to the regulation of both energy homeostasis and glucose homeostasis (Levin 2001; Penicaud, Leloup et al. 2002). The detection of β cell glucose sensing components (GK, GLUT2, ATP-sensitive K channel) in the hypothalamus has led to the hypothesis that hypothalamus uses a similar model as pancreatic β cells for glucose sensing (Schuit, Huypens et al. 2001). The β cell glucose sensor glucokinase has been proposed to be a likely glucose sensor in the hypothalamus (Dunn-Meynell, Routh et al. 2002). The evidence that GLUT2 is expressed in the brain, and blocking brain GLUT2 has affected energy homeostasis suggests a possible role of GLUT2 in the brain glucose sensing, leading to the hypothesis for this work.

Hypotheses

The hypothalamus and the AP/NTS area in the hindbrain are involved in glucose sensing and regulation of food intake (Ritter, Slusser et al. 1981; Penicaud, Pajot et al. 1990; Navarro, Rodriquez de Fonseca et al. 1996; Lynch, Tompkins et al. 2000; Schuit, Huypens et al. 2001). Peripherally, GLUT2 is one of the glucose sensors in the pancreatic beta cells and is required for glucose stimulated insulin secretion. GLUT2 has been found expressed in the hypothalamus and the hindbrain (Brant, Jess et al. 1993; Leloup, Arluison et al. 1994); GLUT2 has been localized in the astrocytes (Leloup, Arluison et al. 1994) and ependymal cells along the third cerebral ventricle (Garcia, Millan et al. 2003), and probably in the neurons as well (Penicaud, Leloup et al. 2002). GLUT2 mRNA in the hypothalamus and the hindbrain is decreased by refeeding in 24-h food restricted rats compared with ad libitum controls (Zhou, Roane et al. 2003); GLUT2 mRNA in the hindbrain in Zucker fatty rats is decreased compared with lean controls (Bogacka, Roane et al. 2004), indicates that brain GLUT2 may be regulated by energy and glycemic status. Antisense oligos blockade of brain GLUT2 results in suppressed food intake and abolished brain regulated insulin secretion

(Leloup, Orosco et al. 1998; Wan, Hulsey et al. 1998), suggesting that brain GLUT2 may be involved in the central glucose sensing. To date, the role of GLUT2 in the brain and the regulation of brain GLUT2 are not completely understood yet.

Based on the role of GLUT2 in the pancreatic β cells, the presence of GLUT2 in the hypothalamus and hindbrain, it is proposed that brain GLUT2 is likely to play a role in the central glucose sensing. It is proposed that GLUT2 in the hypothalamus and hindbrain will be upregulated by low energy and low glucose status, which will be tested in long term underfed rats and in cultured brain tissue as well as in the GLUT2-expressing N1E-115 neuroblastoma cell line. To investigate the role of brain GLUT2, GLUT2 will be overexpressed in the GT1-7 neuroblastoma cell line, and it is proposed that GLUT2 overexpression in the cells will result in higher cellular energy status and lower AgRP mRNA expression, compared with control cells. Taken together, this dissertation will demonstrate the involvement of GLUT2 in brain glucose sensing and possibly regulation of food intake.

CHAPTER 3

DISTRIBUTION OF GLUCOKINASE, GLUCOSE TRANSPORTER GLUT2, SULFONYLUREA RECEPTOR-1, GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AND NEUROPEPTIDE Y MESSENGER RNAS IN RAT BRAIN BY QUANTITATIVE REAL TIME RT-PCR[1]

Introduction

GK, GLUT2, SUR1, GLP-1R and NPY have been identified in a number of brain areas (Gehlert, Chronwall et al. 1987; Morris 1989; Leloup, Arluison et al. 1994; Navarro, Rodriquez de Fonseca et al. 1996; Karschin, Ecke et al. 1997; Merchenthaler, Lane et al. 1999; Lynch, Tompkins et al. 2000; Dunn-Meynell, Routh et al. 2002). GK has been proposed as a mediator of glucose sensing in the brain (Schuit, Huypens et al. 2001; Dunn-Meynell, Routh et al. 2002). GLUT2 has been proposed to play a role in central regulation of food intake (Wan, Hulsey et al. 1998) or glucose sensing (Leloup, Orosco et al. 1998). SUR1 may be important in central glucose sensing mechanisms (Lee, Dixon et al. 1999). GLP-1R and NPY have been associated with central inhibition and stimulation of food intake, respectively (Navarro, Rodriquez de Fonseca et al. 1996; Turton, O'Shea et al. 1996; Schwartz, Woods et al. 2000). GLP-1R mRNA is colocalized with GK or GLUT2 in the hypothalamus (Navarro, Rodriquez de Fonseca et al. 1996), NPY mRNA is colocalized with GK in the arcuate nucleus (ARC) (Lynch, Tompkins et al. 2000), implying that the regulation of GLP-1R or NPY activity may be influenced by glucose sensing processes. In the present study, the mRNA expression of GK, GLUT2, SUR1, GLP-1R and NPY in ten brain areas from individual rats is quantified using real time RT-PCR. Such information has not been previously reported and could be valuable as an initial step to understanding the central functions of these molecules.

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Materials and Methods

Materials and Reagents

RNA extraction kit was obtained from Stratagene (La Jolla, CA). Taqman Primers and probes for GLUT2, AgRP and cyclophilin were obtained from Biosearch Technologies (Novato, CA). Real time RT-PCR reagents including MuLV reverse transcriptase, RNAse inhibitor and PCR reagent were obtained from Applied Biosystems (Branchburg, NJ).

Brain Micropunch and RNA Isolation

Four male S5B rats, two months old, were used in this study. Coronal brain sections were prepared at 300 um using a cryostat. In the hindbrain, sections were collected with three rostral to and two caudal to bregma –13.30 mm where the central canal disappears and the fourth ventricle appears. In the hypothalamus, seven sections were collected rostral to bregma –3.30 mm where the medial eminence appears (Paxinos and Waston 1998). Nucleus of the solitary tract (NTS), area postrema (AP), hypoglossal nucleus (12), inferior olive (IO), lateral reticular nucleus (LRt), ARC, ventromedial nucleus (VMN), paraventricular nucleus (PVN) and lateral hypothalamic area (LH) were removed by micropunch (Palkovits and Brownstein 1988). Care was taken not to include the ependymal cells around the third ventricle. Cortex samples were collected from the same sections containing ARC as a control. Total RNA from each area of each rat was isolated following the manual of RNA extraction kit, and RNA quantity was determined by OD_{260}.

Real Time RT-PCR

Primers and Taqman probes (Table 1) for cyclophilin, GK, GLUT2, SUR1, GLP-1R, and NPY were synthesized by Biosearch Technologies. Cyclophilin was used as an internal control. Real time RT-PCR was performed on an ABI PRISM 7700 sequence detector. The reaction mixture contained: 10 ng RNA, PCR buffer, 5.5 mM $MgCl_2$, dATP, dCTP, dTTP

Table 1. Primer and probe sequences for real-time RT-PCR. Cyc, cyclophilin; GK, glucokinase; GLUT2, glucose transporter GLUT2; SUR1, sulfonylurea receptor-1; GLP-1R, glucagon-like peptide-1 receptor; NPY, neuropeptide Y; F, forward primer; R, reverse primer; P, TaqMan probe.

Name	Sequence (5'-3')	Genbank
Cyc	F: CCCACCGTGTTCTTCGACAT R: TGCAAACAGCTCGAAGCAGA P: CAAGGGCTCGCCATCAGCCG	M19533
GK	F: CAAGCTGCACCCGAGCTT R: TGATTCGATGAAGGTGATTTCG P: TCAGCCTGCGCACACTGGCG	M25807
Glut2	F: GTCCAGAAAGCCCCAGATACC R: TGCCCCTTAGTCTTTTCAAGCT P: TTGCCCTGACTTCCTCTTCCAAATTTAGGTAA	NM_012879
SUR1	F: CACATTCACCACAGCACCTG R: CCAGCTGGCATGTATAAGTG P: CCCGTCAGACAGGATACCCTCAG	NM_013039
GLP-1R	F: CTGCATCGTGATAGCCAAGCT R: GGACTTCGCGAGTCTGCATT P: AGGCTAATCTCATGTGTAAGACC	S75952
NPY	F: TCTGCCTGTCCCACCAATG R: CAACGACAACAAGGGAAATGG P: CCACCACCAGGCTGGATTCCGA	M20373

and dGTP each 0.3 mM, 500 nM forward primers, 500 nM reverse primers, 200 nm Taqman probes, 5 U RNAse inhibitor, 12.5 U MuLV Reverse Transcriptase, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The reaction condition was: 48°C for 30 min for RT; one cycle of 95°C for 10 min plus 40 cycles of 95°C for 15 sec and 60°C for 1 min for PCR. PCR products were sequenced. The relative mRNA level of each gene was expressed as its ratio to cyclophilin. Data are reported as means ± SE.

Results

Sequencing of PCR products confirmed the correct amplification of cyclophilin, GK, GLUT2, SUR1, GLP-1R and NPY mRNA. GK mRNA was expressed at high levels in ARC and VMN, moderate in PVN, LH and IO, low in NTS, AP, LRt, very low in 12 and not detected in cortex. GLUT2 mRNA was expressed at high levels in 12, moderate to low in NTS, AP, IO, LRt, ARC, VMN, PVN, LH, and very low in cortex. SUR1 mRNA was expressed at moderate to low levels across the hindbrain and the hypothalamus, and at moderate levels in cortex. GLP-1R mRNA was expressed at low levels in the hypothalamus and the hindbrain, with relatively higher expression in AP and ARC, and not detectable in cortex. NPY mRNA expression was highest in ARC, moderate in NTS, AP, IO, LRt and VMN, low in PVN and LH, and very low in 12. Moderate levels of NPY mRNA were detected in cortex (Figure 1).

Discussion

In the current study, we have demonstrated mRNA distribution of GK, GLUT2, SUR1, GLP-1R and NPY in rat brain. Real time RT-PCR, together with micropunch, provides a sensitive and reliable method for examination of mRNA expression in specific brain areas. Our results are consistent with previous in situ hybridization and conventional RT-PCR studies

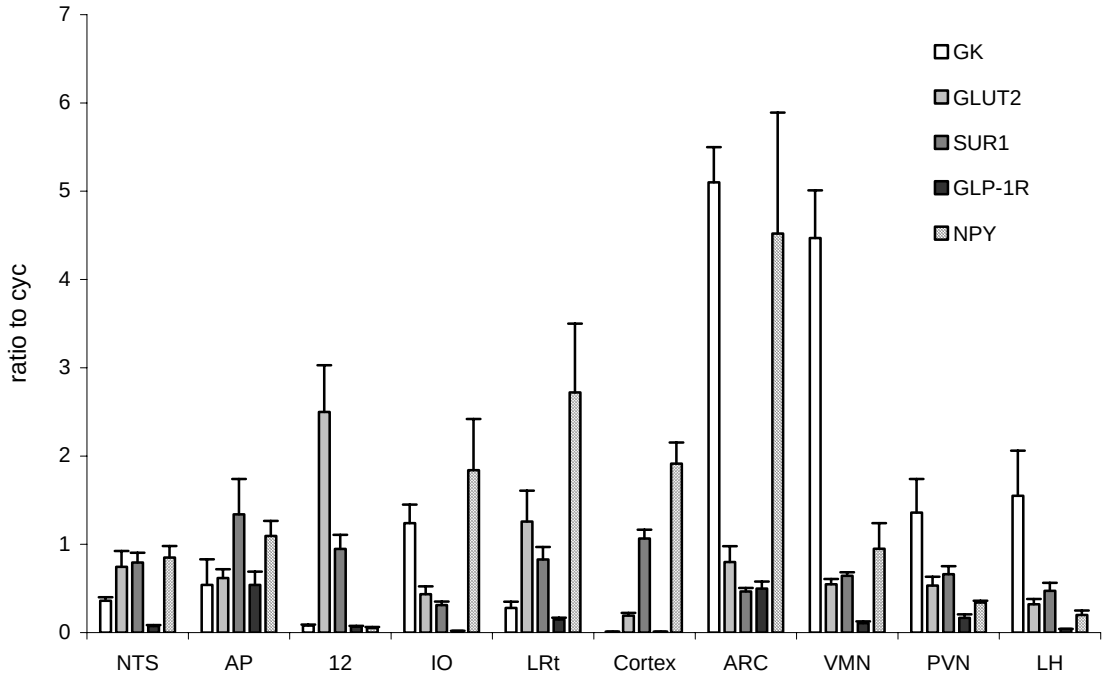

Figure 1. Glucokinase (GK), glucose transporter GLUT2, sulfonylurea receptor-1 (SUR1), glucagon-like peptide-1 receptor (GLP-1R) and neuropeptide Y (NPY) mRNA expression in specific areas in S5B rat brain. The relative amount of mRNA was expressed as ratio to cyclophilin (cyc). NTS, nucleus of the solitary tract; AP, area postrema; 12, hypoglossal nucleus; IO, inferior olive; LRt, lateral reticular nucleus; ARC, arcuate nucleus; VMN, ventromedial nucleus; PVN, paraventricular nucleus; LH, lateral hypothalamic area. Data are reported as means ± SE.

(Gehlert, Chronwall et al. 1987; Morris 1989; Leloup, Arluison et al. 1994; Navarro, Rodriquez de Fonseca et al. 1996; Karschin, Ecke et al. 1997; Merchenthaler, Lane et al. 1999; Lynch, Tompkins et al. 2000; Dunn-Meynell, Routh et al. 2002). GK has been proposed to mediate glucose sensing in the brain (Dunn-Meynell, Routh et al. 2002); GLUT2 has been proposed to play a role in central regulation of food intake (Wan, Hulsey et al. 1998) or glucose sensing (Leloup, Orosco et al. 1998). The presence of GK and GLUT2 mRNA in ARC, VMN, PVN, LH, NTS and AP indicates that GK and GLUT2 may function in these areas that are known to be involved in central glucose sensing and regulation of food intake (Contreras, Fox et al. 1982; Bird, Cardone et al. 1983; Ritter, Dinh et al. 2000; Schuit, Huypens et al. 2001; Dunn-Meynell, Routh et al. 2002). IO, LRt and 12 have not been related to glucose sensing and the function of GK and GLUT2 mRNA in these nuclei is unknown. It is interesting to note that GLUT2 mRNA distribution in the brain is not parallel to GK. GLUT2 mRNA was lower than GK in ARC, VMN, PVN, LH and IO, similar to GK in AP, and higher than GK in NTS, 12 and LRt. GLUT2 has only been identified in astrocytes (Leloup, Arluison et al. 1994), and GK appears to be primarily expressed in neurons (Lynch, Tompkins et al. 2000; Dunn-Meynell, Routh et al. 2002), which might explain their non-parallel expression. On the other hand, these brain areas might assume different roles in terms of glucose sensing, as is seen in pancreatic α and β cells, where α cells only express GK but not GLUT2, whereas β cells express both (Heimberg, De Vos et al. 1995; Heimberg, De Vos et al. 1996). SUR1 is the regulatory subunit of ATP-sensitive K^+ (K_{ATP}) channels; the widespread expression of SUR1 mRNA is in agreement with the two proposed roles of K_{ATP} channels in the brain: neuroprotection and glucose sensing when co-expressed with other glucose sensing mediators (Lee, Dixon et al. 1999; Mobbs, Kow et al. 2001). The relatively high expression of GLP-1R mRNA in ARC and AP suggests that ARC and AP may be the

major sites of GLP-1 activity. The high levels of NPY mRNA in ARC is consistent with previous reports that ARC is the predominant nucleus for NPY synthesis (Schwartz, Woods et al. 2000). Whether NPY mRNA in IO and LRt is related to food intake regulation or other functions as a neurotransmitter is yet to be elucidated.

CHAPTER 4

THE ROLE OF GLUCOSE TRANSPORTER 2 IN BRAIN GLUCOSE SENSING

Introduction

Glucose transporter 2 (GLUT2) is a high Km and low affinity facilitative glucose transporter. GLUT2 has been identified in both the peripheral tissue and the brain. In the peripheral tissue, GLUT2 has been localized in liver, pancreatic β cell, intestine and kidney (Thorens, Sarkar et al. 1988). In the liver, GLUT2 is the major glucose transporter that is responsible to transport glucose in and out of hepatocytes to maintain blood glucose homeostasis (Thorens, Sarkar et al. 1988). Liver GLUT2 is decreased by fasting and increased by refeeding (Thorens, Flier et al. 1990). Liver GLUT2 is up regulated by glucose, which requires glucose metabolism (Asano, Katagiri et al. 1992; Rencurel, Waeber et al. 1996). In the pancreatic β cells, GLUT2 has been proposed as a glucose sensor and GLUT2 is required for glucose stimulated insulin secretion (Guillam, Hummler et al. 1997; Guillam, Dupraz et al. 2000). β cell GLUT2 is decreased during hypoglycemia and increased during hyperglycemia (Thorens, Deriaz et al. 1996). As in the liver, β cell GLUT2 is upregulated by glucose, which also requires glucose metabolism (Ferrer, Gomis et al. 1993).

In the brain, GLUT2 has been found to be widely expressed at low levels (Brant, Jess et al. 1993; Leloup, Arluison et al. 1994). Compared with the cortex, GLUT2 expression in specific areas in the hypothalamus and hindbrain are relatively higher (Leloup, Arluison et al. 1994; Li, Xi et al. 2003). In the brain, GLUT2 has been localized in the astrocytes (Leloup, Arluison et al. 1994) and ependymal cells along the third cerebral ventricle (Garcia, Millan et al. 2003); and GLUT2 may be expressed in the neurons as well (Penicaud, Leloup et al. 2002). Antisense oligos blocking of brain GLUT2 resulted in suppressed food intake,

increased energy expenditure and abolished brain regulated insulin secretion (Leloup, Orosco et al. 1998; Wan, Hulsey et al. 1998), suggesting that brain GLUT2 might be involved in the central glucose sensing. GLUT2 mRNA in the hypothalamus and the hindbrain is decreased by refeeding in 24 hour food restricted rats compared with ad libitum controls (Zhou, Roane et al. 2003); GLUT2 mRNA in the hindbrain in Zucker fatty rats is decreased compared with lean controls (Bogacka, Roane et al. 2004), indicates that brain GLUT2 may be regulated by energy and glycemic status. To date, the role of GLUT2 in the brain and the regulation of brain GLUT2 are not completely understood yet.

In our previous report, we have used N1E-115 and GT1-7 neuroblastoma cell lines as in vitro model to investigate the effect of glucose on cellular ATP levels and orexigenic neuropeptide AgRP mRNA expression (Lee, Li et al. 2005). In this study, we investigated the regulation of brain GLUT2 by energy status in the two-week underfed rats; we applied various glucose to rat AP/NTS tissue culture and GLUT2-expressing N1E-115 cell culture to understand the role of glucose in the regulation of brain GLUT2. To illustrate the function of GLUT2 in the brain, we overexpressed rat liver GLUT2 in the GT1-7 cells; and examined cellular ATP and AgRP mRNA response to various glucose and 2DG in the GLUT2 overexpressed GT1-7 cells and control cells.

Materials and Methods

Materials and Reagents

For glucose measurement: glucose reagent (Sigma, St. Louis, MO). For tissue culture, cell culture and treatments: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA (Invitrogen, Carlsbad, CA), glucose and 2-deoxyglucose (2DG) (Sigma, St. Louis, MO). For RNA isolation from micropunched brain tissue: RNA extraction kit (Stratagene, La Jolla, CA). For RNA isolation from tissue

and cell culture: Trizol (Invitrogen, Carlsbad, California), chloroform (Fisher Scientific, Fair Lawn, NJ). isoproponal and formamide (Sigma, St. Louis, MO). For GLUT2 cloning: Access RT-PCR kit, restriction enzyme KpnI, NotI, BamHI and HindIII (Promega, Madison, WI), PCR purification kit and plasmid purification kit (Qiagen, Santa Clarita, CA), TA cloning kit and pcDNA3.1 vector (Invitrogen, Carlsbad, CA). For transfection and selection: LipofectAMINE, OptiMEM medium and Geneticin G418 (Invitrogen, Carlsbad, CA). For Western Blot: protease inhibitor (Sigma, St. Louis, MO), rabbit anti-GLUT2 antibody (Biogenensis, Poole, England), HRP-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA), and chemiluminent reagent (Periece, Rockford, IL). For ATP measurement: ATP Assay Kit (Sigma, St. Louis, MO). For real time RT-PCR: Taqman Primers and probes for GLUT2, AgRP and cyclophilin (Biosearch Technologies, Novato, CA), real time RT-PCR reagents including MuLV reverse transcriptase, RNAse inhibitor and PCR reagent (Applied Biosystems, Branchburg, NJ).

Animal

Male Sprague-Dawley rats (Harlan, Indianapolis IN), were housed in hanging cages in a temperature (22 ± 2°C) and humidity (40-50%) controlled room, with a 12-h light:dark cycle. Rats had free access to tap water and regular rat chow (Lab Diet, rat chow 5001) unless otherwise specified. Animal care and use was carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Louisiana State University and Pennington Biomedical Research Center.

Male Sprague-Dawley rats, weighing about 300 g, were fed powder rat chow (Lab Diet, rat chow 5001). The animals were randomly assigned to two groups (n = 8 each group): ad libitum fed or given 50% of ad libitum food intake for two weeks. Body weight was recorded daily in the morning. At the end of the study, the animals were euthanized by

decapitation. For serum sample, trunk blood was collected, placed in ice, allowed to clot, centrifuged at 6000 rpm for 20 min at 4°C, serum was collected and stored at -70 °C. For brain, whole brain was excised and placed on dry ice, and later stored at -70 °C.

Serum Glucose Measurement

Serum glucose was measured using glucose reagent according to the manufacturer's instructions. To make the standard curve for glucose, 60 mg, 120 mg, 180 mg, 240 mg and 300 mg of glucose in 3 ul of water was mixed with 300 ul of glucose reagent, incubated at 37°C for 3 min, and OD340 was determined. The OD340 values were regressed on the glucose concentration to obtain the glucose standard curve. For sample measurement, 3 ul of sample serum was mixed with glucose reagent, incubated at 37°C for 3 min, and OD340 was determined. The glucose concentration in each sample was calculated based on the standard curve, expressed as mmol/l (mM).

Brain Micropunch and RNA Isolation

Coronal brain sections were prepared at 300 um using a cryostat. In the hindbrain, bregma –13.30 mm where the central canal disappears and the fourth ventricle appears was used as a recognition marker; and three sections rostral to plus two sections caudal to bregma –13.30 mm were collected. In the hypothalamus, seven sections were collected rostral to bregma –3.30 mm where the medial eminence appears (Paxinos and Waston 1998). The sections were thaw-mounted on a glass slide, and stored on dry ice. Nucleus of the solitary tract (NTS), area postrema (AP), arcuate nucleus (ARC), ventromedial nucleus (VMN), paraventricular nucleus (PVN) and lateral hypothalamic area (LH) were removed by micropunch (Palkovits and Brownstein 1988). Care was taken not to include the ependymal cells along the third ventricle. The inner diameter of the punch was 0.52 mm. After each punch, the tissue was immediately put into 100 ul of ice-cold 2-mecaptoethanol-lysis buffer

and vortex vigorously for 20 to 30 sec until homogenized. Two tubes of lysis buffer were used alternatively for one rat and always kept on ice during the interim of vortex to prevent overheating. Equal volumes of 70% ethanol were added to the tissue lysate and mixed thoroughly by vortexing for 5 sec. The ethanol-lysate mixture was stored at -70°C until we completed the punching of all the samples before proceeding with RNA isolation.

Total RNA from micro-punched tissue was isolated from each individual rat following the manual of the RNA extraction kit. Two ethanol-lysate mixtures from one rat were vortexed again for 5 sec, combined and transferred to a RNA-binding cup that was placed on a 2-ml collection tube, and centrifuged for 1 min at 18,000 x g at 4°C. The following spin also occurred at 18,000 x g at 4°C. The filtrate was discarded, and 600 ul of low-salt wash buffer was added to the cup, which was then spun for 1 min. The filtrate was discarded and the cup was spun for another 2 min to dry the fiber matrix. Next, 30 ul of RNase-free DNase I solution was applied directly onto the fiber matrix inside the cup, which was then incubated in a 37°C water bath for 15 min. The RNA was washed sequentially with 500 ul of high-salt, 600 ul and 300 ul of low-salt wash buffer; after each wash the cup was spun for 1 min. Following the final wash the cup was spun for 2 min to dry the fiber matrix. The cup was transferred to a 1.5-ml collection tube, and 40 ul of elution buffer was added directly onto the fiber matrix. After a 2-min incubation at room temperature, the cup was spun for 1 min. The eluted RNA was mixed with 20 ul of formamide and stored at -70°C. For RNA quantification, 10 ul of sample RNA was diluted into 90 ul of H_2O, and the optical density (OD) 260 and OD280 was measured using a UV spectrophotometer. GLUT2 mRNA expression was determined by real time RT-PCR. For each brain area, GLUT2 mRNA levels were normalized by cyclophilin and reported as percentages of values, considering the values obtained from the ad libitum fed rats in the respective area as 100 percentages.

Ex Vivo Culture

Male adult Sprague Dawley rats, weighing about 400 g, were euthanized by decapitation. Fresh AP/NTS area in the hindbrain were excised (from bregma –14.60 mm to -13.00 mm) (Paxinos and Waston 1998) and immediately cultured in the DMEM containing 2% FBS, and 1 mM or 2.5 mM glucose (n = 11 for each treatment) at 37°C for 3 h. The tissue was homogenized in 500 ul of Trizol, total RNA was isolated following the manufacturer's instructions. The homogenate-chloroform was mixed with 100 ul of chloroform and centrifuged at 18,000 x g at 4°C for 15 min for phase separation. For RNA precipitation, 150 ul of aqueous supernatant was transferred to a 1.5 ml-Eppendorf tube, mixed with 200 ul of isoproponal, and incubated at room temperature for 15 min, followed by centrifuge at 18,000 x g at 4°C for 15 min. The precipitated RNA pellet was washed by 300 ul of 75% ethanol, centrifuged at 18,000 x g at 4°C for 5 min, the ethanol was removed, and the RNA pellet was allowed to dry in air for 5 to 10 min. The RNA pellet was dissolved in 40 ul of RNase-free H_2O containing 50% formamide and stored at -70°C. GLUT2 mRNA expression was determined by real time RT-PCR. GLUT2 levels were normalized by cyclophilin and reported as percentages of values, considering the values obtained at 2.5 mM glucose as 100 percentages.

In Vitro Culture and Treatment of N1E-115 Cells

N1E-115 mouse neuroblastoma cell line was obtained from ATCC (Manassas, VA). The cells were grown in DMEM containing 25 mM glucose, 10% fetal bovine serum (FBS), 50 units of penicillin and 50 µg/ml streptomycin at 37°C in 5% CO_2 unless otherwise specified. For glucose treatment, N1E-115 cells were grown to 90% confluence, and then incubated in DMEM containing 2% FBS and various levels (1, 2.5, 5 mM) of glucose for 16 h. The treatment was repeated three times with duplicate each time. The cells were

homogenized in 500 ul of Trizol, total RNA was isolated as stated in *ex vivo* culture, and dissolved in 40 ul of RNase-free H_2O containing 50% formamide. GLUT2 mRNA expression was determined by real time RT-PCR. GLUT2 levels were normalized by cyclophilin and reported as percentages of values, considering the values obtained at 2.5 mM glucose as 100 percentages.

Real Time RT-PCR for GLUT2

Real time RT-PCR was used to determine GLUT2 mRNA levels in the samples; cyclophilin (cyc) was used as an internal control to normalize the RNA levels in each sample. The sequences of the primers and probes for rat cyclophilin and GLUT2, mouse cyclophilin and GLUT2 were listed in Table 2. Real time RT-PCR reaction mixture was 50 ul of total volume, including 10 ng of sample RNA, 1 X PCR buffer, 5.5 mM MgCl2, dATP, dCTP, dUTP and dGTP each 0.3 mM, 500 nM forward primers, 500 nM reverse primers, 200 nM Taqman probes, 5 U RNase inhibitor, 12.5 U MuLV reverse transcriptase, 1.5 U AmpliTaq Gold DNA polymerase and RNase-free H_2O. Each sample was tested in duplicate. Reverse transcription was carried out at 48°C for 30 min. PCR was carried out at 95°C for 10 min for one cycle, followed by 40 cycles of 95°C for 15 sec and 60^{o}C for 1 min. Data was output as Ct number (cycle threshold number) and the data within the linear region of the amplification curve are analyzed according to ABI's user bulletin #2. The relative amounts of GLUT2 mRNA were calculated using ΔΔCt method and normalized by cyclophilin.

Mammalian Cell Expressing Vector pcDNA3.1-GLUT2

Full-length rat GLUT2 (Genbank NM_012879) cDNA was obtained by RT-PCR using Access RT-PCR kit from Promega. The primers used to amplify GLUT2 were: forward 5'TCCGCACACAACATGTCAGAA3', reverse 5'TCCTCACACAGTCTCTGATG3'. The RT-PCR reaction was performed in a volume of 50 ul, containing (in final concentration) 1 x

Table 2. The sequences of primers and probes for real time RT-PCR. F: forward primer, R: reverse primer, P: Taqman probe, cyc: cyclophilin, GLUT2, glucose transporter 2, AgRP: agouti-related peptide.

Gene	Sequence	Genbank
Rat cyc	F: 5'CCCACCGTGTTCTTCGACAT3' R: 5'TGCAAACAGCTCGAAGCAGA3' P: 5'CAAGGGCTCGCCATCAGCCG3'	M19533
Rat GLUT2	F: 5'GTCCAGAAAGCCCCAGATACC3' R: 5'TGCCCCTTAGTCTTTTCAAGCT3' P: 5'TTGCCCTGACTTCCTCTTCCAAATTTAGGTAA3'	NM_012879
Mouse cyc	F: 5'GGCCGATGACGAGCCC3' R: 5'TGTCTTTGGAACTTTGTCTGCAA3' P: 5'TGGGCCGCGTCTCCTTCGA3'	NM_008907
Mouse GLUT2	F: 5'GACAAACTTGGAAGGATCAAAG3' R: 5'AATTTGGAACATCCCATCAAGAG3' P: 5'CTGCAAACAGCCTCTCATTGACTGGAGC3'	NM_031197
Mouse AgRP	F: 5'GTACCGCCACGAACCTCTGT3' R: 5'TCCCCTGCCTTTCCCAAA3' P: 5'TCGCACCTAGCCAATGGATGTT3'	NM_007427

AMV/Tfl buffer, 1 mM MgSO4, 0.2 mM dATP, dCTP, dDTP and dGTP each, 1 uM forward and reverse primer each, 5 units of AMV reverse transcriptase, 5 units of Tfl DNA polymerase, and 0.5 ug of liver RNA as template. RT-PCR reaction was carried out at 45°C for 45 min; 94°C for 2 min; 39 cycles of 94°C for 30 sec, 58°C for 1 min, and 68°C for 2 min and 30 sec; and final extension at 68°C for 10 min. PCR products were detected by 1% agarose gel. The ~1.6 kb PCR band of GLUT2 was excised from the gel, purified by PCR purification kit, and cloned into pCR2.1 vector (TA cloning kit). The insertion of GLUT2 gene in pCR2.1 vector was verified by HindIII and BamHI restriction enzyme digestion, and further sequenced to ensure correct open reading frame of GLUT2. To transfer the GLUT2 gene from pCR2.1 vector to mammalian cell expressing pcDNA3.1 vector, the pcDNA3.1 and pCR2.1-GLUT2 vector were subject to KpnI / NotI restriction enzyme digestion, which was verified by 1% agarose gel. The ~1.6 kb GLUT2 cDNA and ~5.4 kb linear pcDNA3.1 were excised from the agarose gel, purified by PCR purification kit, and subject to ligation. The GLUT2 insertion into pcDNA3.1 was verified by EcoRI enzyme digestion. The pcDNA3.1 vector with correct GLUT2 insert, referred to as pcDNA3.1-GLUT2, was amplified, purified by plasmid purification kit, quantified by OD260, and used for in vitro transfection.

In Vitro Transfection of GT1-7 Cells with pcDNA3.1-GLUT2 Vector

GT1-7 mouse neuroblastoma cell line was a generous gift from Dr. Pamela Mellon (University of California, San Diego). The cells were grown in DMEM containing 25 mM glucose, 10% FBS, 50 units of penicillin and 50 μg/ml streptomycin at 37°C in 5% CO_2. On the day before transfection, the cell culture medium was changed to DMEM containing 25 mM glucose and 10% FBS but without antibiotics. On the day of transfection, GT1-7 cells were about 50-70% confluence. For one well of cells grown in a 6-well plate, 4 ug of

pcDNA3.1-GLUT2 vector and 10 ul of transfection reagent LipofectAMINE were diluted with 250 ul of OptiMEM medium each. The diluted DNA and LipofectAMINE were mixed gently and the mixture was incubated at room temperature for 20 min. GT1-7 cells were incubated with DNA-LipofectAMINE mixture for 4 h to allow LipofectAMINE mediated DNA entry into the cells, then fresh DMEM containing 25 mM glucose and 10% FBS was added. The next day, the cells were split at 1:10 ratio, and subject to selection with 500 ug/ml G418 for 10 –14 days. The pcDNA3.1 vector contain G418-resistant gene; by G418 selection, only cells with the vector incorporated into the genome that were able to express G418 resistant gene could survive. In the end, G418-resistant clones of cells were pooled and amplified for subsequent treatments. In parallel, GT1-7 transfected with pcDNA3.1 empty vector were obtained to serve as controls in the following experiments. In the following experiments, GT1-7 cells transfected with pcDNA3.1-GLUT2 vector will be referred to as GT1-7_{GLUT2}, and GT1-7 cells transfected with pcDNA3.1 empty vector will be referred to as GT1-7_{pcDNA}. The stably transfected cells were grown in DMEM containing 25 mM glucose, 10% FBS, 50 units of penicillin, 50 μg/ml streptomycin and 250 ug/ml G418.

Western Blot of GLUT2

The overexpression of GLUT2 in GT1-7 cells was verified by Western Blot. The cells were homogenized in lysis buffer containing 100 mM Tris-HCl, pH 6.8, 0.1% SDS and 1% protease inhibitor. The cell lysate was centrifuged at 10,000 rpm for 10 min at 4°C, and supernatant was collected for protein detection. The protein was separated on an 8% SDS-polyacrylamyl gel and transferred to PVDF membrane. Non-specific bindings were blocked with 5% non-fat dry milk in TBST buffer (10 mM Tris-HCl, pH at 7.4, 150 mM NaCl, 0.5% Tween-20) for 1 h. The membrane was incubated with polyclonal rabbit anti-GLUT2 primary antibodies for 3 h (1:1000 dilution), and washed five times for 10 min each. The membrane was further incubated with horseradish

peroxidase (HRP) conjugated secondary antibody (1:10,000 dilution) for 1 h, and washed five times for 10 min each. The antibodies were diluted in 5% non-fat dry milk in TBST buffer, the membrane was washed in TBST buffer, and the incubation and washes were carried out at room temperature. The protein on the membrane was detected by chemiluminent reagent.

Treatments of GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells

To examine AgRP mRNA response to glucose, GT1-7_{GLUT2} and GT1-7_{pcDNA} cells were cultured in DMEM containing 2% FBS and various levels of glucose (1, 2.5, 5, 10, 25 mM) for 16 or 24 h. To examine AgRP mRNA response to 2DG, GT1-7_{GLUT2} and GT1-7_{pcDNA} cells were cultured in DMEM containing 2% FBS, 2.5 mM glucose with or without 5 mM 2DG for 24 h. Total RNA was isolated using Trizol, the mRNA expression of AgRP was determined by real time RT-PCR (methods were described above.). Primers and probes for AgRP were listed in Table 2. Data were expressed as ratio to cyclophilin, and were reported as percentages of values. For glucose dose response, we considered the values obtained at 1 mM glucose of each group as 100 percentages, for response to 2DG, we considered the values obtained at 2.5 mM glucose without 2DG treatment of each group as 100 percentages.

ATP Measurement

Cellular ATP concentration (n = 3-6) was measured using the ATP Assay Kit according to the manufacturer's instructions. 300 ul of ATP lysis buffer were used for Cells cultured in 24-well plate to release ATP; 50 ul of cell lysate were used for reaction with an equal amount of diluted ATP assay mix for ATP measurement. Data were reported as percentages of values, considering the values obtained at 1 or 2.5 mM glucose without 2DG as 100 percentages for glucose dose response or response to 2DG experiment, respectively.

Data Analysis

Data were presented as mean ± S.E.M. For underfeeding and *ex vivo* studies, differences between the means were assessed by Student's t-test. For in vitro study, differences between the means were assessed by Student's t-test or one-way analysis of variance (ANOVA) and Tukey's test. The significance level was set at $p < 0.05$.

Results

Two-Week Underfeeding Study

At the end of two-week feeding, the body weight gain was - 65.3 ± 1.8 g for the underfed rats, and 37.9 ± 4.4 g for the ad libitum fed rats (Figure 2). The body weight gain was significantly different between the underfed and ad libitum fed rats ($p < 0.0001$, t-test). The serum glucose levels were 6.47 ± 0.21 mM for the underfed rats, and 6.02 ± 0.08 mM for the ad libitum fed rats. The serum glucose levels were not significantly different between the two groups of rats.

Relative GLUT2 mRNA expression in the AP, NTS, ARC, VMN, PVN and LH was determined by real time RT-PCR; in each area, GLUT2 mRNA levels in the ad libitum fed rats were considered as 100 percentages (Figure 3). GLUT2 mRNA was significantly increased in the AP in two-week underfed rats, compared with ad libitum fed rats ($p < 0.05$, t-test). In the hypothalamic arcuate nucleus, GLUT2 mRNA was slightly increased in underfed rats but did not reach statistical significance. In other hypothalamic and hindbrain areas examined, GLUT2 mRNA was not different between the underfed rats and ad libitum fed rats.

Ex Vivo Culture

The AP/NTS area was excised from the rat brain, and cultured in 1 or 2.5 mM glucose at 37˚C for 3 h. As shown in Figure 4, the GLUT2 mRNA had a significant 25%

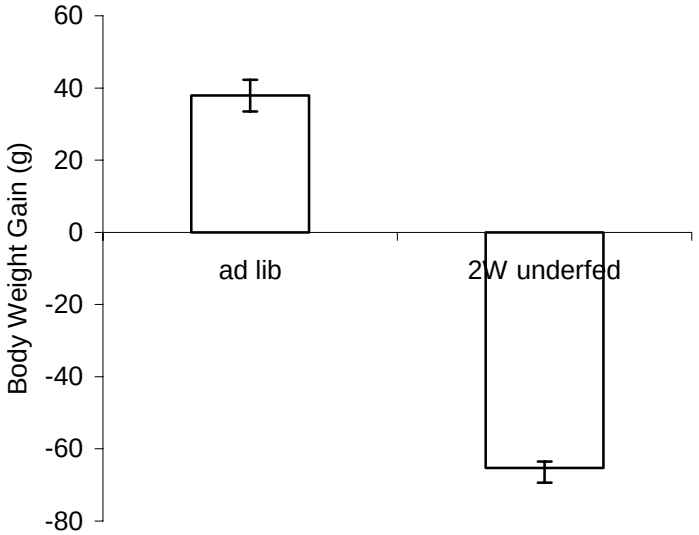

Figure 2. Body weight gain (g) in the two-week 50% underfed rats and ad libitum fed rats. The body weight gain was significantly different between underfed rats and ad libitum fed rats ($p < 0.0001$).

increase in AP/NTS tissue cultured in 1 mM glucose compared with those cultured in 2.5 mM glucose ($p = 0.03$, t-test).

In Vitro Cell Culture

N1E-115 cells were grown in DMEM containing 25 mM glucose and 10% FBS till 90% confluence, then switched to DMEM containing 2% FBS and 1, 2.5 or 5 mM glucose for 16 h. As shown in Figure 5, in N1E-115 cells, GLUT2 mRNA was decreased by glucose ($p = 0.014$, One-way ANOVA). GLUT2 mRNA levels were significantly lower when N1E-115 cells were incubated with 5 mM glucose compared with 1 mM glucose ($p < 0.05$, Tukey's test).

Overexpression of GLUT2 in GT1-7 Cells

PCR2.1-GLUT2 vector was sequenced and confirmed to contain the correct in-frame GLUT2 gene as described in Genbank. After transfection of pcDNA3.1-GLUT2 or pcDNA3.1 vector into GT1-7 cells, the overexpression of GLUT2 protein was verified by Western Blot. GT1-7 cells do not express endogenous GLUT2 protein; GT1-7 cells transfected with pcDNA3.1-GLUT2 vector express high levels of GLUT2 protein; liver protein was used as a positive control (Figure 6).

Glucose Treatment of GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells

After 16 h of incubation with glucose, cellular ATP levels were measured in GT1-7_{GLUT2} cells and GT1-7_{pcDNA} cells. As shown in Figure 7, glucose significantly increased cellular ATP in both GT1-7_{GLUT2} ($p < 0.0001$, one-way ANOVA) and GT1-7_{pcDNA} cells ($p < 0.001$, one-way ANOVA). In GT1-7_{pcDNA} cells, ATP levels were significantly higher at 2.5 mM glucose compared with 1 mM glucose, increasing glucose from 2.5 mM to 5, 10 and 25 mM could not further increase ATP levels ($p < 0.05$, 1 mM vs. 2.5, 5, 10 and 25 mM

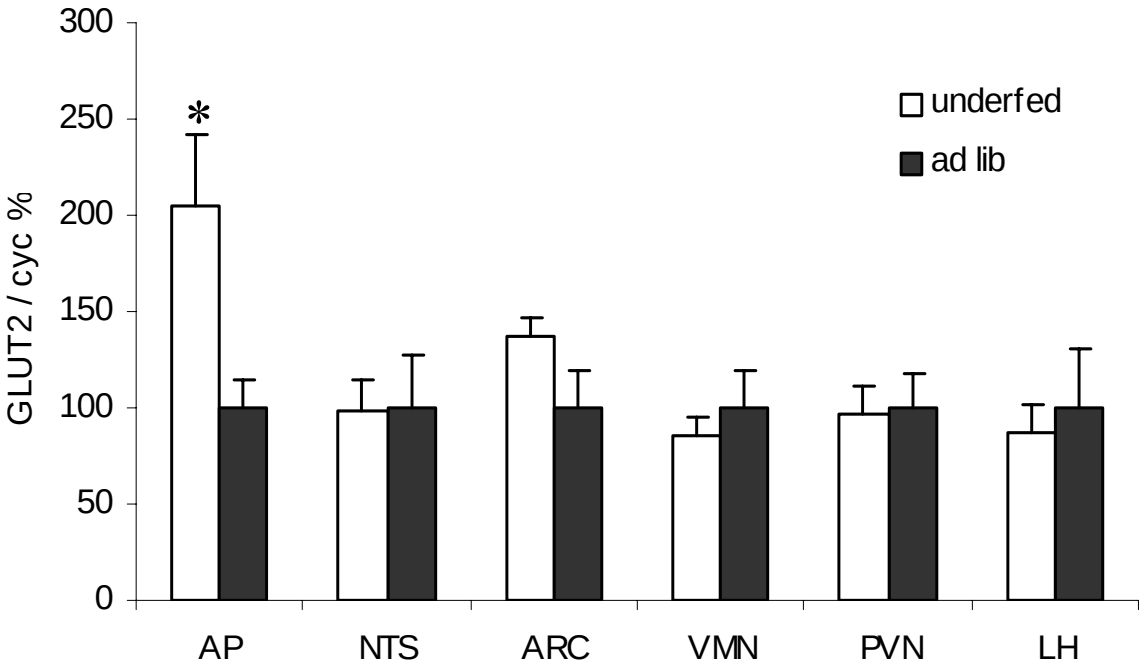

Figure 3. GLUT2 mRNA expression in the AP, NTS, ARC, VMN, PVN and LH in the two-week 50% underfed rats and ad libitum fed rats. GLUT2 mRNA was significantly increased in the AP in underefed rats ($p < 0.05$).

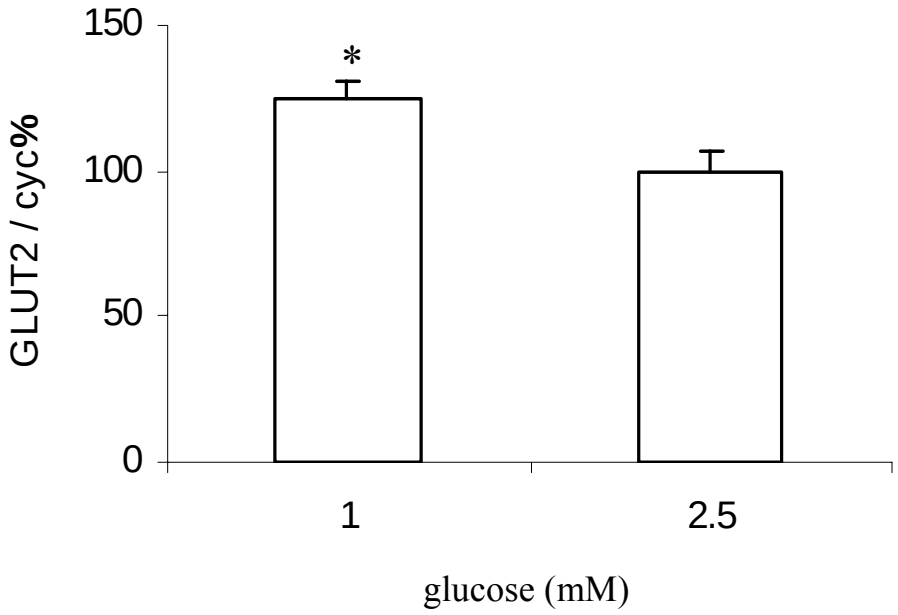

Figure 4. GLUT2 mRNA response to glucose in *ex vivo* cultured AP/NTS tissue. Rat AP/NTS tissue was dissected and cultured in DMEM plus 2% FBS and 1 or 2.5 mM glucose at 37˚C for 3 h. GLUT2 mRNA in AP/NTS tissue was significantly increased by 1 mM glucose compared with 2.5 mM glucose ($p < 0.05$).

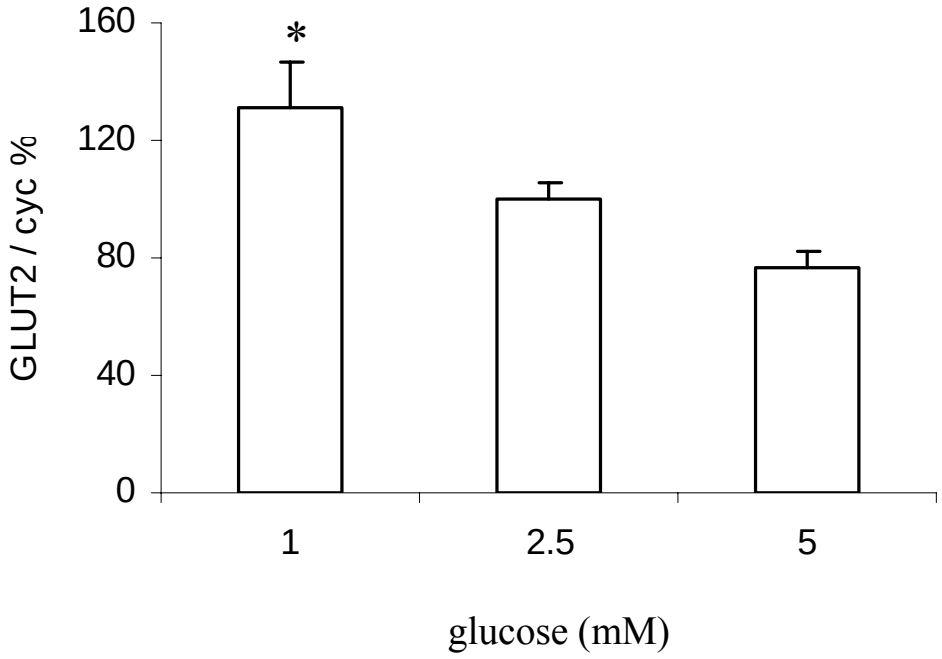

Figure 5. GLUT2 mRNA response to glucose in N1E-115 cell cutlute. N1E-115 cells were cultured in DMEM plus 2% FBS and 1, 2.5 and 5 mM glucose at 37˚C, 5% CO2 for 16 h. GLUT2 mRNA in N1E-115 cells was significantly decreased by glucose ($p < 0.05$); GLUT2 mRNA was significantly higher at 1 mM glucose compared with 5 mM glucose ($p < 0.05$).

1 2 3

Figure 6. Western Blot showing overexpression of GLUT2 protein in GT1-7 cells. Lane 1: GT1-7_{GLUT2} cells; lane 2: GT1-7_{pcDNA} cells; lane 3: rat liver control.

glucose, Tukey's test). In GT1-7_{GLUT2} cells, ATP levels were significantly increased in a dose-dependent manner from 1 to 5 mM glucose, increasing glucose from 5 mM to 10 and 25 mM could not further increase ATP levels ($p < 0.05$, 1 mM vs. 2.5, 5, 10 and 25 mM glucose, 2.5 mM vs. 5, 10 and 25 mM glucose, Tukey's test). At 5, 10 and 25 mM glucose, ATP levels in GT1-7_{GLUT2} cells were significantly higher compared with GT1-7_{pcDNA} cells ($p < 0.001$, t-test).

AgRP mRNA response to glucose in GT1-7_{GLUT2} and GT1-7_{pcDNMA} cells were shown in Figure 8. Glucose significantly decreased AgRP mRNA expression in both GT1-7_{pcDNA} ($p < 0.0001$, one-way ANOVA) and GT1-7_{GLUT2} cells ($p = 0.0007$, one-way ANOVA). At 25 mM glucose, AgRP mRNA was lowered to 56% of values at 1 mM glucose in GT1-7_{pcDNA} cells, whereas AgRP mRNA was lowered to 37% of values at 1 mM glucose in GT1-7_{GLUT2} cells. AgRP mRNA in GT1-7_{GLUT2} cells had a more sensitive response to higher glucose (25 mM) compared with GT1-7_{pcDNA} cells ($p < 0.05$, AgRP percentage value at 25 mM in GT1-7_{GLUT2} vs GT1-7_{pcDNA} cells, t-test).

2DG Treatment of GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells

In GT1-7_{pcDNA} cells, 2DG induced about 200% increase in AgRP mRNA compared with no 2DG control ($p = 0.0003$, t-test). In GT1-7_{GLUT2} cells, 2DG induced about 35% increase in AgRP mRNA compared with no 2DG control ($p = 0.0019$, t-test). 2DG-stimulated increase in AgRP mRNA in GT1-7_{GLUT2} cells was significantly smaller compared with GT1-7_{pcDNA} cells ($p = 0.003$, t-test) (Figure 9A). After 24-h incubation with 2DG, the cellular ATP levels decreased by 40.2% ($p < 0.0001$, t-test) and 35.9% ($p < 0.001$, t-test) in GT1-7_{pcDNA} and GT1-7_{GLUT2} cells, respectively, compared with no 2DG controls. 2DG induced depletion of cellular ATP was similar in both GT1-7_{GLUT2} and GT1-7_{pcDNA} cells (Figure 9B).

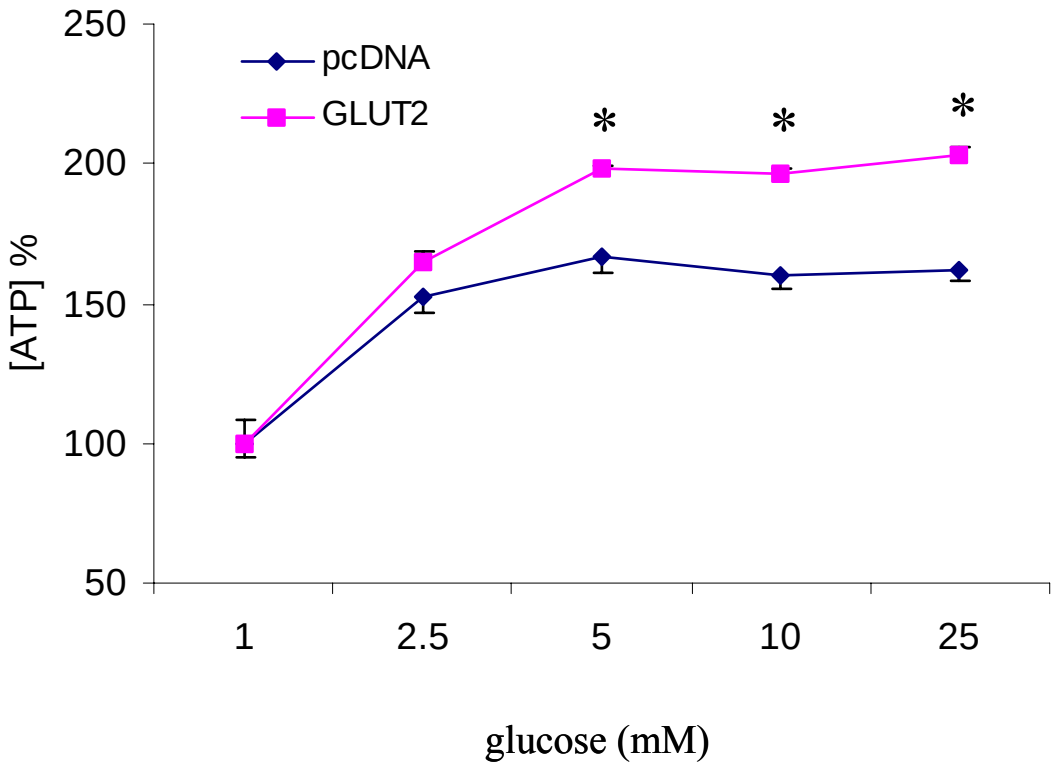

Figure 7. Cellular ATP response to glucose in GT1-7_{GLUT2} and GT1-7_{pcDNA} cells. GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells were cultured in DMEM plus 2% FBS and 1, 2.5, 5, 10 and 25 mM glucose at 37˚C, 5% CO2 for 16 h. Glucose significantly increased cellular ATP levels in both GT1-7_{GLUT2} ($p < 0.0001$) and GT1-7_{pcDNA} cells ($p < 0.001$). At 5, 10 and 25 mM glucose, cellular ATP in GT1-7_{GLUT2} cells was significantly higher compared with GT1-7_{pcDNA} cells ($p < 0.001$).

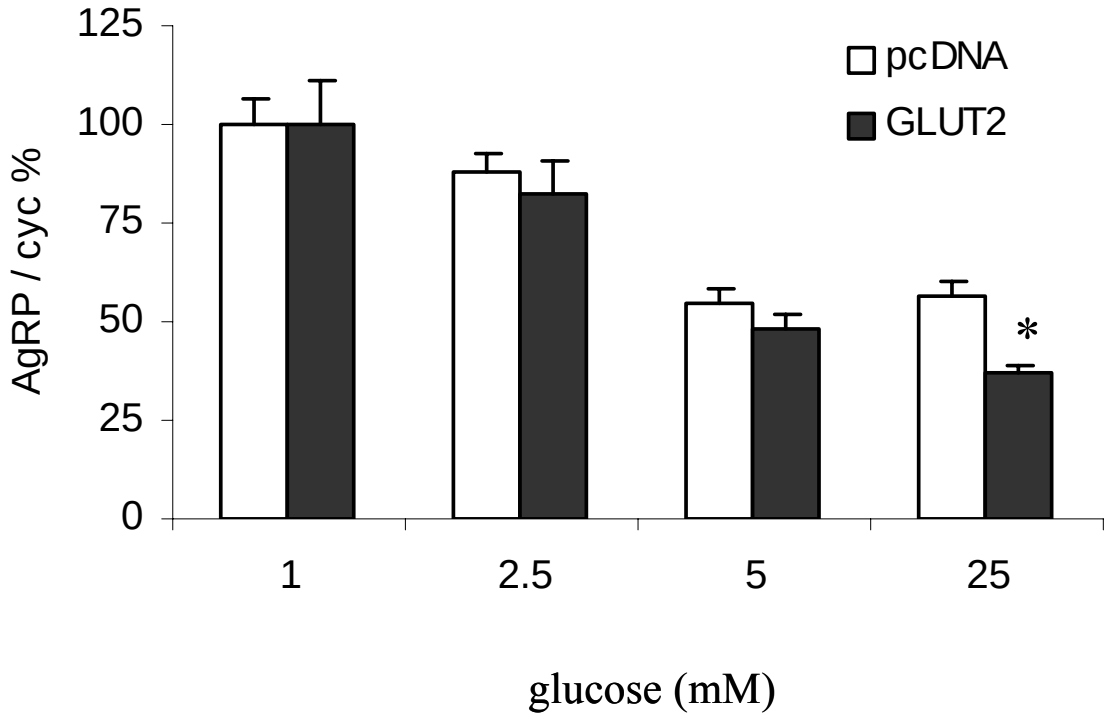

Figure 8. AgRP mRNA response to glucose in GT1-7_{GLUT2} and GT1-7_{pcDNA} cells. GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells were cultured in DMEM plus 2% FBS and 1, 2.5, 5, 10 and 25 mM glucose at 37˚C, 5% CO2 for 24 h. Glucose significantly decreased AgRP mRNA in both GT1-7_{GLUT2} ($p < 0.001$) and GT1-7_{pcDNA} cells ($p < 0.0001$). At 25 mM glucose, AgRP mRNA was significantly lower in GT1-7_{GLUT2} cells compared with GT1-7_{pcDNA} cells ($p < 0.05$).

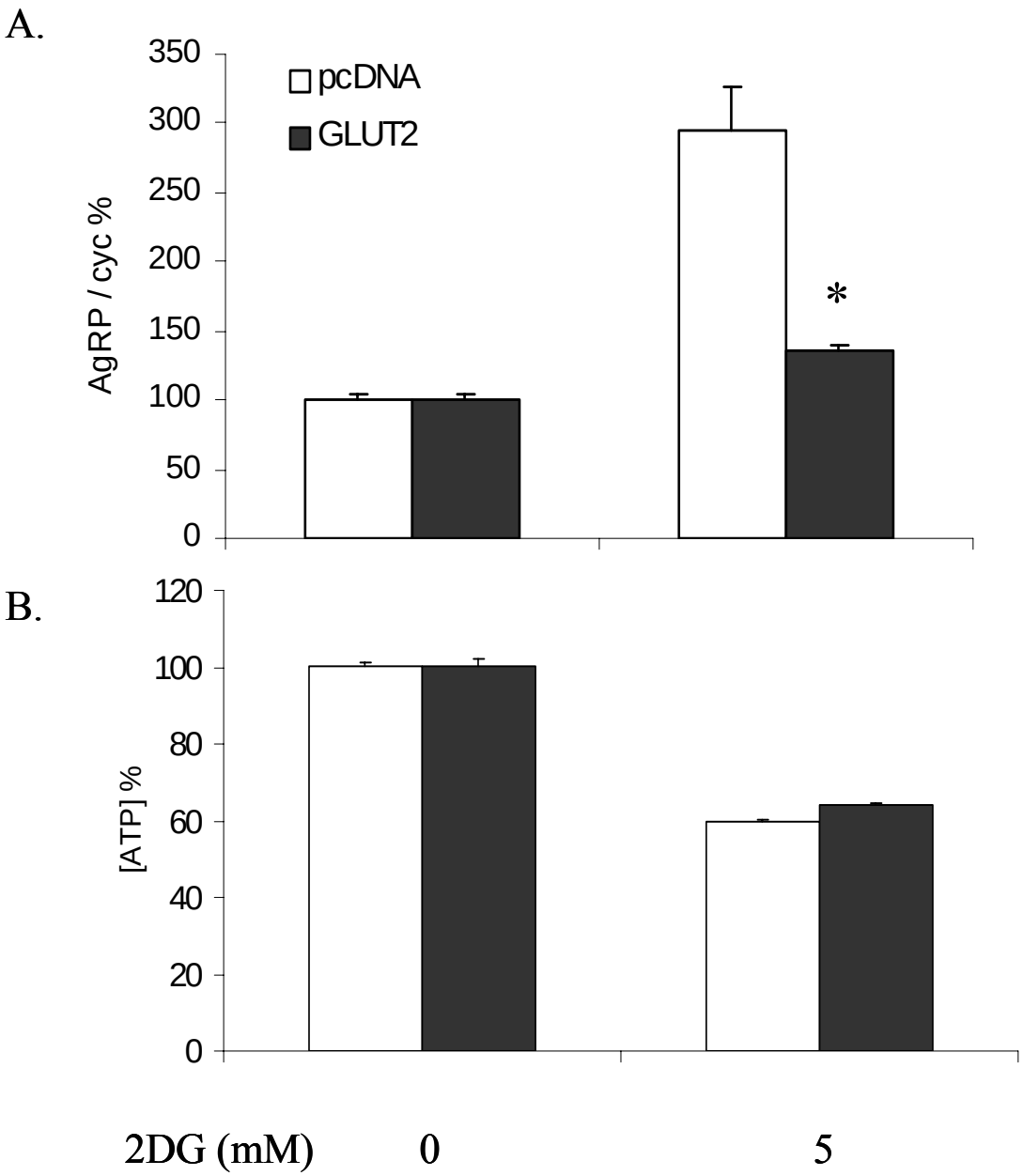

Figure 9. AgRP mRNA and cellular ATP responses to 2DG in GT1-7_{GLUT2} and GT1-7_{pcDNA} cells. GT1-7_{GLUT2} and GT1-7_{pcDNA} Cells were cultured in DMEM, 2% FBS, 2.5 mM glucose with or without 5 mM 2DG at 37˚C, 5% CO2 for 24 h. A. AgRP mRNA was significantly increased by 2DG in both GT1-7_{GLUT2} ($p < 0.01$) and GT1-7_{pcDNA} cells ($p < 0.001$). 2DG-induced AgRP mRNA was significantly less in GT1-7_{GLUT2} cells compared with GT1-7_{pcDNA} cells ($p < 0.01$). B. 2DG significantly decreased cellular ATP levels in both GT1-7_{GLUT2} ($p <0.001$) and GT1-7_{pcDNA} ($p < 0.0001$) cells. 2DG induced similar depletion of cellular ATP in both cells.

Discussion

In this study, we investigated the effect of energy status on brain GLUT2 expression and the effect of GLUT2 overexpression on energy status in neuronal cells. We found that in contrary to the peripheral tissue such as liver and pancreatic islets, brain GLUT2 mRNA was upregulated by low energy status. In vivo, we mimicked low energy status by 50% underfeeding rats for two weeks, during which period the animals lost more than 60 g of body weight compared with 40 g of body weight gain in the ad libitum fed controls. Serum glucose measured at the end of the experiment was not different between the two groups; however, one time point of blood glucose does not represent the fluctuation of blood glucose during the whole experiment, and it is apparent that body weight is a better index of low energy status than blood glucose here. In the underfed rats, GLUT2 mRNA was increased in the hindbrain area postrema compared with ad libitum fed rats. In *ex vivo* and *in vitro*, we mimicked low energy status and low glucose status by culturing tissue or cells in 1 mM glucose. In cultured rat AP/NTS tissue, GLUT2 mRNA was increased by 1 mM glucose compared with 2.5 mM glucose; in N1E-115 cells, GLUT2 mRNA was increased by 1 mM glucose compared with 5 mM glucose. The results from *in vivo*, *ex vivo* and *in vitro* consistently demonstrated that brain GLUT2 mRNA was upregulated by low energy status, which is opposite to peripheral tissue. In the liver and pancreatic islets, GLUT2 was decreased by fasting and increased by refeeding (Chen, Alam et al. 1990; Thorens, Flier et al. 1990). In primary hepatocyte culture and isolated pancreatic islet culture, GLUT2 mRNA and protein were increased by high glucose such as 27.8 mM for hepatocytes and 16.7 mM for islets (Asano, Katagiri et al. 1992; Ferrer, Gomis et al. 1993; Rencurel, Waeber et al. 1996). Previous work from our group had shown that GLUT2 mRNA was decreased in the hindbrain in Zucker fatty rats compared with lean controls (Bogacka, Roane et al. 2004); 1-h

refeeding reduced GLUT2 mRNA in both the hindbrain and the hypothalamus in 24-h 50% food restricted rats (Zhou, Roane et al. 2003), indicating that GLUT2 in the brain was downregulated by hyperlycemia or refeeding. The results from this study provided additional evidence to demonstrate that GLUT2 mRNA in the hindbrain area postrema was upregulated in long term underfed rats. Furthermore, we demonstrated that in AP/NTS tissue culture and in cell culture, GLUT2 mRNA was increased by low glucose (1 mM) compared with 2.5 or 5 mM glucose, implicating that brain GLUT2 mRNA is directly regulated by glucose. Particularly, we have shown that lowering glucose concentrations does-dependently decreased intracellular ATP levels and simultaneously increased AgRP mRNA expression in the N1E-115 cell culture (Lee, Li et al. 2005). The neuronal cells detected the low energy status reflected by decreased ATP levels, and increased AgRP mRNA, which would have stimulated feeding *in vivo*. In the meantime, the neuronal cells increase the high Km GLUT2 expression, and other molecules such as glucokinase (unpublished data), in order to increase the ability to process elevated glucose associated with feeding. In this sense, GLUT2 may play a role the brain glucose sensing.

Of the six hypothalamic and hindbrain areas examined, only area postrema showed increased GLUT2 mRNA in underfed rats compared with control, suggesting that area postrema may play an important role in glucose sensing particularly sensing hunger. Previous studies have demonstrated that hindbrain but not the hypothalamus is essential for glucoprivation induced feeding. Injection of 5TG into the lateral cerebral ventricle failed to simulate feeding after blocking aqueduct connecting the third and fourth ventricle, whereas injection of 5TG into the fourth ventricle could still stimulate feeding, indicating that the hindbrain was necessary for stimulation of glucoprivic feeding (Ritter, Slusser et al. 1981; Penicaud, Pajot et al. 1990). Lesions of area postrema in the hindbrain blocked 5TG or 2DG

induced feeding, further suggesting that the area postrema is a critical area for glucoprivic feeding (Contreras, Fox et al. 1982; Bird, Cardone et al. 1983). Injection of 2DG or 5TG into the arcuate nucleus, ventromedial nucleus, paraventricular nucleus or lateral hypothalamus did not induce feeding and hyperglycemia (Miselis and Epstein 1975; Ritter, Dinh et al. 2000). In contrast, injection of 5TG into the nucleus of the solitary tract, dorsomedial and ventrolateral medulla induced feeding and hyperglycemia (Ritter, Dinh et al. 2000). Taken together, hindbrain is necessary for response to glucoprivation. Our results are in agreement with these studies to implicate that hindbrain is more important for glucose sensing under low energy status than the hypothalamus.

Next we investigated the effect of GLUT2 overexpression in neuronal cells, and found that GLUT2 overexpression resulted in a 20% elevation in cellular ATP when the cells were incubated with ambient glucose (5, 10, 25 mM) in the medium. GLUT3, which has a high affinity and low Km for glucose, is the major glucose transporter in neuronal tissues (Olson and Pessin 1996). At 5 mM and higher glucose levels, GLUT3 has already been saturated and intracellular glucose cannot further increase in proportional to extracellular glucose levels. Thus, glucose transport becomes rate limiting for glucose usage by neuronal cells, which is reflected by saturation of cellular ATP and glucose induced inhibition of AgRP at higher glucose levels. GLUT2 has a low affinity and high Km for glucose (Olson and Pessin 1996); after overexpressing GLUT2 in the neuronal cells, glucose transport is no longer rate limiting for glucose usage by the neuronal cells. GLUT2 overexpression allows increased glucose flux through glycolysis to generate ATP, resulting in higher cellular ATP levels and greater inhibition of AgRP at high glucose levels (25 mM glucose). Consistent with previous report, glucose dose dependently increases cellular ATP and decreases AgRP mRNA expression in both GT1-7_{GLUT2} cells and control GT12-7_{pcDNA} cells (Lee, Li et al.

2005). The increase of ATP and decrease of AgRP mRNA by glucose was not strictly parallel, which suggests that ATP is not the only factor that affects AgRP expression, AMP-activated protein kinase (Minokoshi, Alquier et al. 2004; Lee, Li et al. 2005) and other unknown mediators may also be involved in the regulation of AgRP expression.

2DG is a glucose analogue that can competitively inhibit glucose metabolism and induce glucoprivation (Brown 1962). GLUT2 overexpression in neuronal cells changes the cellular response not only to high glucose, but also to 2DG. Compared with GT1-7_{pcDNA} cells, in which 2DG induced about 2-fold increase in AgRP mRNA expression, 2DG only stimulated 35% increase in AgRP mRNA in GT1-7_{GLUT2} cells. In contrast, 2DG induced similar depletion of cellular ATP in both cells. The induction of AgRP and depletion of ATP by 2DG treatment is consistent with our previous report, in which we have shown that 2DG increased AgRP mRNA in neuronal cell culture and *ex vivo* cultured mouse hypothalamus. We also showed that 2DG exerted the glucoprivic effect by decreasing cellular energy status, which is supported by the findings that 2DG dose-dependently reduced cellular ATP and supplement of pyruvate partially reversed the effect of 2DG on ATP and AgRP mRNA (Lee, Li et al. 2004). However, the discrepancy between the similar ATP depletion and significantly different stimulation of AgRP mRNA in both cells after 2DG treatment indicates that AgRP mRNA in the neuronal cells is also regulated by factors other than ATP, such factors could be AMP-activated protein kinase and probably factors unknown at present (Minokoshi, Alquier et al. 2004; Lee, Li et al. 2005). More work is needed before we can explain why GLUT2 overexpression attenuated 2DG stimulated AgRP mRNA in the neuronal cells.

Previous study has reported that ICV injection of antisense oligos to GLUT2 mRNA (daily injection for 13 days) suppressed cumulative food intake and diminished the feeding

response to 2DG in rats compared with missense control (Wan, Hulsey et al. 1998). In another study, injection of antisense oligos to GLUT2 mRNA directly into the arcuate nucleus (twice per day for two days) reduced body weight without changing food intake, as well as eliminated insulin response induced by carotid artery injection of a small amount of glucose (Leloup, Orosco et al. 1998). These two studies showed that blocking brain GLUT2 mRNA decreased food intake, increased energy expenditure or impaired brain-regulated insulin secretion. However, these studies neither confirmed that brain GLUT2 protein was reduced, nor identified in which brain area GLUT2 protein was reduced after antisense treatment. In addition, neither study examined the mechanism of how blocking GLUT2 mRNA reduced food intake, such as whether arcuate neuropeptide was involved or due to other mechanisms. Our study showed that GLUT2 overexpression led to greater inhibition of AgRP in the presence of high glucose. Based on the *in vitro* study, we predict that overexpression of GLUT2 in the brain will lead to decreased hypothalamic AgRP expression and therefore decreased food intake, which appears to be contradictory to the conclusions of the above two studies. However, the results from *in vivo* study might be opposite to *in vitro* study due to the complex feedback, the adaptation systems in the animals, and the timing of the measurements. Unless the effect of GLUT2 overexpression in the brain and the involved mechanisms are examined, it is too early to speculate why both blocking and overexpressing GLUT2 in the brain appear to produce similar effect on food intake.

In this study, we provide evidence to indicate that GLUT2 is likely to play a role in the brain glucose sensing. We showed that brain GLUT2 mRNA was upregulated in response to low energy status (particularly to low glucose), and overexpresssion of GLUT2 increased energy status in neuronal cells, reflected by increased intracellular ATP levels and decreased hunger signals (AgRP mRNA) at high glucose concentrations, and attenuated AgRP response

to glucoprivation. Further work is needed to confirm if overexpression of GLUT2 in the brain will similarly enhance cellular energy status, suppress hunger signals and thereby reduce food intake in the animal studies.

CHAPTER 5

CONCLUSIONS

The work in this dissertation includes two parts. In the first part, we combined tissue micropunch technique and real time RT-PCR to quantify relative mRNA levels of multiple genes in specific brain nuclei. We demonstrated glucokinase (GK), glucose transporter GLUT2, sulfonylurea receptor-1 (SUR1), glucagon-like peptide-1 receptor (GLP-1R) and neuropeptide Y (NPY) mRNA expression in nine areas in the rat hypothalamus and hindbrain. The colocalization of GK, GLUT2, SUR1 and GLP-1R in the same areas in the hypothalamus and hindbrain implicates the presence of β cell glucose sensing mechanism in the brain; the colocalization of these genes with NPY in the arcuate nucleus suggests that the brain glucose sensing may play a role in the regulation of food intake.

In the second part of this dissertation, we focused on the regulation of brain GLUT2 by energy status and the effect of GLUT2 overexpression on energy status in neuronal cells. We showed that brain GLUT2 mRNA was upregulated in response to low energy status (particularly low glucose status) under three conditions: *in vivo*, *ex vivo* and *in vitro*. We further showed that overexpresssion of GLUT2 increased energy status in neuronal cells, decreased hunger signals (AgRP mRNA) at high glucose concentrations, and attenuated hunger signals (AgRP mRNA) induced by glucoprivation. We provide evidence to indicate that GLUT2 is likely to play a role in the brain glucose sensing, and brain glucose sensing appears to participate in the regulation of food intake.

Further work is needed to confirm if overexpression of GLUT2 in the brain will similarly enhance cellular energy status, suppress hunger signals and thereby reduce food intake in the animal studies. The presence of other components of β cell glucose sensing

apparatus in the brain suggests that brain may use similar mechanism as β cells for glucose sensing; and instead of regulation of insulin secretion, brain glucose sensing may be involved in the regulation of energy homeostasis. Similar work on GK, another β cell glucose sensor, can be done as the work on GLUT2 in this dissertation, in order to understand better the mechanisms of brain glucose sensing and their involvement in the regulation of energy homeostasis.

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ELSEVIER

APPENDIX

LETTER OF PERMISSION

23 November 2004 Our ref: HG/HDN/NOV04/J140

Bing Li
C/O Mr Roy J Martin
Neurobiobehaviour Laboratory
Pennington Biomedical Research
Baton Rouge, 70802
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Dear Mr Martin

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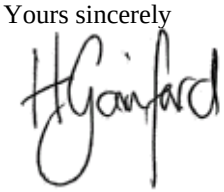

Helen Gainford
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VITA

Bing Li was born on April 26, 1971, in TianJin, People's Republic of China. She attended elementary school from 1978 to 1984, middle school from 1984 to 1987, and high school from 1987 to 1990. In 1990, she was admitted into the seven-year program of TianJin Medical University to study medicine. She spent the first two years in the Department of Biology, NanKai University, for pre-med study, the next four years in TianJin Medical University for medical study, and the last year in The Department of Endocrinology, General Hospital of TianJin Medical University working on her thesis. In 1997, she graduated from TianJin Medical University as Master of Medicine. From 1997 to 2000, she worked in the Department of Medical Microbiology in TianJin Medical University as an instructor. She taught medical microbiology and at the same time was involved in a couple of research projects. To improve herself in the academic and research field, she went back to school for doctoral study starting from June 2000. Majoring in nutrition, she followed Dr Roy J Martin in U.S. as a graduate student, in the Department of Foods and Nutrition at the University of Georgia for the first year, and in the School of Human Ecology at Louisiana State University for the rest of her advanced study. Upon graduation, she will work in a postdoctoral position in related fields for several years. She hopes she will develop into a scientific researcher and an educator in the future.